Enzyme Assay Shopping/Planning

Project: Duke iGEM 2016
Authors: evmiles
Date: 2016-06-08

WEDNESDAY, 6/8/16

Emma: TAX10

- **Benzoyl coenzyme A lithium salt**
  - $235 for 5 mg
  - http://www.sigmaaldrich.com/catalog/product/sigma/b1638?lang=en&region=US
- Coupled Reaction for the other substrate
  - BAPT?
- Ellman's Reagent
  - $298 ish (prices vary)
  - http://www.sigmaaldrich.com/catalog/search?interface=All&term=ellmans%20reagent&N=0&mode=match%20partialmax&focus=product&lang=en&region=US&cm_re=Did%20You%20Mean-_-ellmans%20reagent-_-elmans%20reagent

Nisa: BadA

SOLUBLE!

- Sodium benzoate
  - $52 for 250 g
  - http://www.sigmaaldrich.com/catalog/search?term=benzoate&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product
- Co-A
  - $300 ish for 100 mg
  - http://www.sigmaaldrich.com/catalog/search?term=Coenzyme+A&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product
- Ellman's Reagent
  - $298 ish (prices vary)
  - http://www.sigmaaldrich.com/catalog/search?interface=All&term=ellmans%20reagent&N=0&mode=match%20partialmax&focus=product&lang=en&region=US&cm_re=Did%20You%20Mean-_-ellmans%20reagent-_-elmans%20reagent

Parth: TycA

- (3R)-β-phenylalanine
  - $86 for 5g
  - http://www.sigmaaldrich.com/catalog/product/aldrich/159492?lang=en&region=US
- Co-A

Thomas: PAM

- (3R)-β-phenylalanine
  - $86 for 5g
  - http://www.sigmaaldrich.com/catalog/product/aldrich/159492?lang=en&region=US
- Phenylalanine Dehyrogenase
  - http://www.sigmaaldrich.com/catalog/product/sigma/p4798?lang=en&region=US
- NAD Sodium Salt Hydrate (Detection of NADH using 340nm light)
  - http://www.sigmaaldrich.com/catalog/product/sigma/n0505?lang=en&region=US
- PBS

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

WEDNESDAY, 6/8/16

Preparation of Stocks for the FGM3, SM++ and SM10 medias

Prepared 1 L of the 10X Ammonium-Citrate 30 in a 2000 mL flask. Autoclaved and immediately poured into an autoclaved 1 L bottle for storage.

Prepared 1 L of the 10X Ammonium-Citrate 90 in a 2000 mL flask. Autoclaved and immediately poured into an autoclaved 1 L bottle for storage.

Prepared the 2M $MgSO_4$ and 10 mM $CaSO_4$ in 500 mL of dH_2O and filter sterilized. Used a 1 L beaker for mixing - mixed on stir plate until completely dissolved.

Also gathered materials for the other solutions.
The other solutions are on hold due to lack of bottles for storage.

Minimal Media 2-Stage Evaluations

Introduction

This standard operating procedure describes the media formulations for preparing media for strain evaluations in various systems as well as the procedures for making concentrated stocks of each media component. All stocks are stored with a green label at room temperature on the second shelf of the cabinet unless otherwise stated.

Materials

- Chemical Reagents as Mentioned in Section 1 of this SOP
- 250 mL to 1000 mL Bottles
- 250 mL to 1000 mL Flasks/Beakers
- Ultrapure Water
- 0.2 µm Filter Units, Varying Volumes
- Autoclave

Procedure

Section 1: Preparation of Stock Solutions

Prepare the stock solutions to the following concentrations using ultrapure water and ACS grade reagents.

1. Prepare 1 liter of a solution of 10X concentrated ammonium-citrate salts, varying components based on the target biomass:

2. Prepare 1 liter of **10X Ammonium-Citrate 30** salts by mixing 30 g of $(NH_4)_2SO_4$ and 1.5 g Citric Acid in water with stirring. Autoclave and store at RT.

3. Prepare 1 liter of **10X Ammonium-Citrate 90** salts by mixing 90 g of $(NH_4)_2SO_4$ and 2.5 g Citric Acid in water with stirring. Autoclave and store at RT.

   Prepare the following buffering reagents:

4. **MOPS Buffer:** Prepare 1 M Potassium 3-(N-morpholino)propanesulfonic Acid (MOPS) and adjust to pH 7.4 with KOH (~40 mL). Filter sterilize (0.2 um) and store at RT.

   Had to add ~ 600 mL of KOH to the media.
   This turned the molarity to **0.625 M**.

5. **Phosphate:** Prepare 0.1 M potassium phosphate buffer, pH 6.8 by mixing 49.7 mL of 1.0 M K_2HPO_4 and 50.3 mL of 1.0 M KH_2PO_4 and adjust to a final volume of 1000 mL with ultrapure water. Filter sterilize (0.2 um) and store at RT.

6. Prepare **2 M $MgSO_4$** and **10 mM $CaSO_4$** solutions. Filter sterilize (0.2 um) and store at RT.

   Dissolve the $MgSO_4$ on a heat block with stirring at around

7. **500X Trace Metals**: Prepare a solution of micronutrients in 1000 mL of water containing 10 mL of concentrated H_2SO_4 : 0.6 g $CoSO_4$*$7H_20$, 5.0 g $CuSO_4$*$5H_20$, 0.6 g $ZnSO_4$*$7H_20$, 0.2 g Na_2MoO_4*$2H_2O$, 0.1 g H_3BO_3, and 0.3 g $MnSO_4$*H_2O. Filter sterilize (0.2 um) and store at RT in the dark.

8. **Iron Sulfate:** Prepare a fresh solution of 40 mM ferrous sulfate heptahydrate in water. Filter sterilize (0.2 um) and discard after 1 day.

9. **Thiamine:** Prepare a 50 g/L solution of thiamine-HCl. Filter sterilize (0.2 um) and store at 4°C.

10. **Glucose:** Prepare a 500 g/L solution of glucose by stirring with heat. Cool, filter sterilize (0.2 um), and store at RT.

Section 2L Final Media Compositions:

11. *Prepare the final working medium by aseptically mixing stock solutions based on the following tables in the order written to minimize precipitation.*

12. **FGM3 Growth and Production Media, pH 6.8:**

    Store in 4C fridge.

Table1

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	3.6	1.80 mM
4	Trace Metals	500 X	2	1 X
5	Fe (II) Sulfate	40 mM	2	0.08 mM
6	MgSO4	2 M	1	2.00 mM
7	CaSO4	10 mM	5	0.05 mM
8	Glucose	500 g/L	90	45 .0 g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	NA	NA
12	Casamino Acids	100 g/L	NA	NA

13. **FGM3 No Phosphate Wash and Production Media, pH 6.8:**

    Store in 4C fridge

Table2

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	0	0
4	Trace Metals	500 X	2	1 X
5	Fe (II) Sulfate	40 mM	2	0.08 mM
6	MgSO4	2 M	1	2.00 mM
7	CaSO4	10 mM	5	0.05 mM
8	Glucose	500 g/L	50	25.0 g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	NA	NA
12	Casamino Acids	100 g/L	NA	NA

14. **SM10 Seed Media, pH 6.8:**

    Store in 4 C fidge

Table3

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.0625 mM
8	Glucose	500 g/L	90	45 .0g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	10	1.0 g/L
12	Casamino Acids	100 g/L	NA	NA

15. **SM10 ++ Seed Media, pH 6.8:**

    Store in 4C fridge

Table4

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.06 mM
8	Glucose	500 g/L	90	45 .0g/L
9	MOPS	1 M	320	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	25	2.5 g/L
12	Casamino Acids	100 g/L	25	2.5 g/L

16. **FGM10 Growth and Production Media, pH 6.8:**

    Store in 4C fridge

Table5

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.06 mM
8	Glucose	500 g/L	50	25 .0 g/L
9	Thiamine-HCl	50 g/L	0.2	0.01 g/L
10	Yeast Extract	100 g/L	NA	NA
11	Casamino Acids	100 g/L	NA	NA

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

THURSDAY, 6/9/16

Redo of the MgSO4 stock

Because the solution yesterday did not filter correctly, a second solution was made using the same protocol.

The solution was heated on a hot plate with stirring for the majority of the day and was left overnight.

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

FRIDAY, 6/10/16

Filter MgSO4

The solution left overnight was mostly dissolved. It was filter sterilized and stored at room temp.

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

TUESDAY, 6/14/16

Rest of Media Stocks

Made stocks of phosphate buffer, KH_2PO_4, K_2HPO_4, 500X Trace Metals, Thiamine, and Glucose.

Thiamine was placed into conical tube and stored in 4C fridge

Also made stock of casamino acids by adding 25 g of casamino acids to 250 mL of pH_2O.

The same method was used to make a stock of yeast. Both the casamino acids and the yeast were autoclaved.

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

FRIDAY, 6/17/16

MOPS Buffer Made

Made MOPS Buffer - had to add ~600 mL of KOH to adjust the pH. Turned molarity to 0.625 M.
Filter sterilized into one 1-L bottle and one 0.5-L bottle.

SM10++ Was Prepped

The SM10++ Media was prepared and mixed minus the the Iron sulfate and thiamine-HCL

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

SUNDAY, 6/19/16

SM10++ Media Finalized

The Iron Sulfate and Thiamine-HCL was added to the SM10++ media. Enough pico water (415 mL) was added to fill to 1L.

The Iron Sulfate was created by dissolving 0.0303 g in 5 mL of pH2O. 4 mL of it was then filtered via syringe into the media.

Stored in 4 C fridge

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

SUNDAY, 6/19/16

Inoculation of BadA and TAT in SM10++

A colony of BadA x 286 and TAT x 286 were inoculated overnight in 50 mL of SM10++
Colonies picked were marked in red with the date on the plate.

Goal: To grow the Samples of 286

Empty Vector Controls Inoculated

TAT-10 (empty vector) was picked from the plate and inoculated in 5 ul of LB+KAN
BadA-A (pGEM033) was inoculated in 5 ul of LB+KAN from the freezer stock.

Goal: To Grow the Empty Vector Control in E-cloni so that the DNA can be gathered.

Phosphate Limited Protein Expression Protocol

Introduction

This standard operating procedure (SOP) describes a protocol for the evaluation of heterologous enzyme expression which is induced by low phosphate conditions.

There are two phases when expressing proteins: (i) growth and (ii) expression. In the first phase, we inoculate media and allow cells to grow and reproduce to reach target cell densities. Once the cells are growing exponentially, we induce for protein production (expression phase).

Protein expression works by activating the promoter associated with your genes of interest (eg. enzyme). There are many different promoters that activate through different signals. In this SOP we use promoters that get activated under low phosphate concentrations. Thus, to induce protein expression (activate a low phosphate inducible promoter), we exchange the media to a media that lacks phosphate.

Once induction has occurred, time is needed for transcription and translation. For protein expression using our system, we evaluate protein expression after at least 16 hours.

When harvesting the cells to assay for protein expression, we centrifuge them and resuspend in a lower volume. This will result in a higher final protein concentration and easier-to-handle volumes.

To extract the proteins (eg enzymes), one needs to lyse the cells. Again, there are many methods available, but we will use sonication. Sonication involves introducing a fine metal tip that vibrates at a high speed. This vibration causes the formation of air bubbles that grow until they explode, creating waves that disrupt cell integrity. Important to note is that sonication causes a lot of heat, so cells need to be lysed in ice. In addition, many cells naturally contain proteases which may degrade your protein, activity of proteases is reduced on ice during lysis. In addition, we may add protease inhibitors to further reduce potential protein degradation.

To confirm production of proteins, one can use sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, a sample of cell lysate is taken and heated briefly at 95 C in the presence of a free thiol reducing agent such as beta-mercaptoethanol or dithiothreitol (DTT) and sodium dodecyl sulfate (SDS). Heat will denature proteins, beta-mercaptoethanol will break disulfide bonds in the protein and SDS which is negatively charged will stick to the denatured proteins and give the proteins an associated number of negative charges based which is based in the size or molecular weight of the protein. Then this mixture of denatured proteins can be run in a polyacrylamide gel exposed to a voltage causing negatively charged species to run through the gel, and since negative charges are added proportionally to the proteins size while bigger proteins get hung up in the gel or run slowere, SDS-PAGE separates proteins based on size. This gel can then be stained to detect protein bands on it. Overexpressed proteins will appear as larger bands.

Materials

- Growth Media: SM10+ Media, pH 6.8, has excess phosphate (1L) –See Appendices Below
- Production Media: SM10-noP Media, pH 6.8 (1L) –See Appendices Below
- Microcentrifuge Tubes
- Disposable Cuvettes
- 10 mL conical centrifuge tubes
- 50 mL conical centrifuge tubes
- 1 mL microcentrifuge tubes
- Spectrophotometer
- Temperature Adjustable Shaking Incubator set to desired temperature (usually 37C)
- 250-mL Non-baffled Shake Flasks
- Antibiotics

- Media Supplements/Induction Chemicals (Thiamine, Anhydrotetracycline, etc.)
- Protease Inhibitor Cocktail (if used)
- Ice bucket and ice
- Sonicator
- SDS-PAGE Components - See SDS Page protocol

Procedure

Day 1:

1. 1. Using Colonies inoculate one new 50 mL culture of each strain to be evaluated in SM10++ defined medium with antibiotics or supplements as required in square culture flasks. Incubate 16 hours in an incubator shaker at 220 rpm and 37 C. Be sure to start an empty vector control cultire.

Day 2:

2. Measure the OD600 of the overnight cultures and record.

   When measuring OD, make sure the values you are getting are between 0 and 0.5. Depending in the particular spectrophotometer, anything above that is not within the linear measuring range of the apparatus and, therefore, is not a reliable measurement. To make sure you are in the linear range, do a 1:10-20 dilution of your culture before measuring the OD. Put this diluted sample in a spectrophotometer cuvette. Set the spectrophotometer to measure absorbance at 600 nm. Fill one cuvette with 1 ml of sterile media or water and blank the spectrophotometer. Then, measure the OD of your sample.

3. Once the cells have reached an OD of 4-6, proceed with below steps.
4. Transfer cultures to 50 mL conical tubes and centrifuge at 4,000 RPM for 20 minutes.
5. Resuspend the cells in 50 mL of FGM3-noP defined medium with antibiotics or supplements as required in square culture flasks. Incubate for hours to allow for protein expression.

Day 3:

6. Transfer cultures to 50 mL conical tubes and centrifuge at 4,000 RPM for 20 minutes.

   This is a good stopping point. You can put the cells in the -80 C freezer. Do not freeze cells that have already been resuspended.

7. Resuspend the cells in 2-5 mL of 50 mM Phosphate buffer pH 8.0 with protease inhibitors and 2 mM EDTA (be mindful that these protease inhibitors or EDTA may inhibit the enzyme you are trying to obtain, so do not add them if that is the case). Transfer the cells in 10 mL conical tubes.

   Best to use 3 ml.
   The lysate buffer can be premade in the lysate buffer protocol.

8. Place the 10 mL conical tubes on ice and sonicate for 3 minutes, 10s-on/30s-off cycles, at 50% tip amplitude (DO NOT increase the amplitude beyond 50%, this is tip specific)
9. Centrifuge down the cell debris at 10,000 rpm at 4 C for 10-15 minutes.
10. Take out and reserve the cell lysate as it should contain your protein of interest (if it is in the soluble fractions, sometime your protein may be expressed but be insoluble so may be spun down or insoluble fraction for analysis as well). Put the cell lysate in 500 uL aliquots in 1 mL microcentrifuge tubes

11. Carefully remove the supernatant into a 30 kDa filter tube

    These are found under where the DNA concentration machine is

12. Spin at 6.7k for 5-10 minutes R

13. Repeat three times adding 200-800ul of lysis buffer or the appropriate buffer for the enzyme assay

    Each time, pipette up and down to prevent the filter from getting clogged.

14. After the last spin, keep the concentrated lower volume lysate.

    The top part will have more liquid than the flow through. This is what is supposed to happen.

15. Take the final liquid on the top and aliqout (800ul) into epi tubes.

16. Add (534 ul) of 50% glycerol to each aliquot

17. Store in -80 C freezer.

18. The cell debris should have twice as much volume of cell debris of 50% glycerol added to it and place into the -80 C freezer.

    We will run a sample of the cell debris on a SDS-PAGE to make sure your protein is not in the insoluble fraction of the cell lysate.

SDS Page Protocol (Protein Gel)

Introduction

This standard operating procedure (SOP) describes a protocol for the detecting changes in protein expression using SDS PAGE.

To confirm expression of proteins, one can use sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, a sample of cell lysate is taken and heated briefly at 95 C in the presence of a free thiol reducing agent such as beta-mercaptoethanol or dithiothreitol (DTT) and (SDS). Heat will denature proteins, beta-mercaptoethanol will break disulfide bonds in the protein and SDS which is negatively charged will stick to the denatured proteins and give the proteins an associated number of negative charges which is based on the size or molecular weight of the protein. Then this mixture of denatured proteins can be run in a polyacrylamide gel exposed to a voltage causing negatively charged species to run through the gel, and since negative charges are added proportionally to the proteins size while bigger proteins get hung up in the gel or run slower, SDS-PAGE separates proteins based on size. This gel can then be stained to detect protein bands on it. Overexpressed proteins will appear as larger bands.

Materials

- Protein Samples
- Bromophenol dye solution (1% w/v)
- 2x Tris-Glycine SDS sample buffer
- SDS-PAGE gel
- Running Buffer
- Protein Gel Box

Procedure

Running the Gel

1. Place the gel inside the gel box, make sure it is a tight fit. Take out the comb to expose the lanes.
2. Pour Tris-SDS running buffer inside the box.
3. Take 10 uL samples of your soluble and insoluble fractions of your cell lysates and mix them with 10 uL of 2x Tris-Glycine SDS sample buffer, which will denature your samples.

   Best to use different dilutions of the lysate.
4. Heat your samples at 95 C for 30-60 seconds, which will further denature your samples.
5. First load 6-10 uL of protein ladder in a well of your SDS-PAGE gel. This is a standard with proteins of known size.
6. Next load 10-15 uL of your samples into new wells of your SDS-PAGE gel.. Make note which lane corresponds to which sample Make note which lane corresponds to which sample.
7. Connect the gel box and run your gel at 140 V for about an hour. The run time may vary, so make sure you do not overrun it. You can track how far it has run by visually tracking the blue dye front as it moves down the gel.
8. Stop the current and carefully take out your gel from the glass plates. Before taking the gel out, you may want to cut a corner of the gel with a razor blade so you can identify the orientation later

9. Stain the gel using Coomassie stain for 1 hour. Do this by placing your gel in a clean dish with enough stain to completely cover the gel.

10. Take out the coomassie stain and reserve (can be reused). Rinse the gel with water.

11. Pour destain solution on your gel and let it destain overnight, Again do this by placing your gel in a clean dish with enough destain solution to completely cover the gel.. Make sure you put aluminum foil over your gel to avoid evaporation.

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

MONDAY, 6/20

FGM3-No P Media was made

The FGM3 - No Phosphate buffer was made using the stocks made earlier. The Iron Sulfate was made by dissolving 0.0303 g in 5 uL of pH2O.

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

MONDAY, 6/20

Inoculated BadA and TAT in FGM3 -No P

Followed Day 2 Instructions to inoculate BadA and TAT in FGM3- No Phosphate

Goal: The samples of 286 need time to express the enzyme

The media does not contain phosphate because the genes are turned on by a lack of phosphate.

Empty Vector Control Mini prepped and Transformed

Goal: Needed the Empty Vector in 286 for Protein Expression

Collected DNA from E. cloni and transformed into 286 cells.

Mini Prep Protocol Zyppy

Electroporation Protocol

Transformations Plated

Plated transformations of empty vector controls into the DLF-00286 strain (5uL of cells, 95 uL of LB)

Samples:

- TAT-10 EV#1
- TAT-10 EV#2
- BadA-A EV#1
- BadA-A EV#2

Results (6/21): Only TAT-10 grew (from now on referred to as EV Control)

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

TUESDAY, 6/21

Lysate Buffer (50ml)

1. Phosphate buffer 2mM
2. EDTA (200ul)
3. Protease inhibiotrs (0.5 pill)
4. water to dilute

Made in a 50 ml conical tube and stored in 4 C fridge.

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

TUESDAY, 6/21

Empty Vector Inoculated in SM10++

Goal: To grow the EV control cells

The empty vector transformation TAT-10 was taken from the plate and inoculated in 50 mL of SM10++.
Placed in shaker overnight and incubated at 37 C.

Made Lysate of BadA and TAT

Day 3 of Phosphate Limited Protein Expression Protocol

1. Transfered the BADA and TAT from FGM-noP to 50ml conicle tubes and brought to lynch lab to spin at 4k rpm for 20 mins
2. Remove/discard the supernatant and resuspended in lysate buffer
3. Lysis using sonicator: room C346 LSRC (two people max)
    a. Pack your sample in a beaker filled with ice so it doesn't move
    b. Use water to wipe off tip.
    c. use the clamp to adjust so the sonicator is above the end of the tube
    d. Set the sonicator using the min/hour setting and put in 10 sec/on and 30 sec off for 3-5 minutes.
    e. Wipe the sonicator between samples. Make sure to turn off and clean before leaving.
    f. Make sure that the samples remain cold when doing a lot of samples.
        I. if need be cool the tip down between samples
4. Spin the lysed cells for 10-15 minutes at 10k rpm
5. carefully remove the supernatant into a 30kDa filter tube from under where the DNA concentration machine is
6. spin at 6.7k for 5-10 minutes and repeat adding 200-800ul of lysis buffer or the appropriate buffer for the enzyme assay
    a. resuspended solid material between spins to avoid filter clogging
7. Take the final liquid on the top and add into aliqouts (800ul) add glycerol if storing at -80C

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

WEDNESDAY, 6/22

Empty Vector Inoculated in FGM3 - No P

Goal: To express protein in the EV Cells

The cells in the SM10++ media was spun down, the SM10++ discarded, and resuspended in the FGM3 media. Shaken and incubated in a flat flask overnight at 37 c.

Day 2 of Phosphate Limited Protein Expression Protocol

Stock Solutions

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-06-08 to 2016-06-23

THURSDAY, 6/23

Made running buffer

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

THURSDAY, 6/23

Ran Gel on TAT, BadA, and EV

Goal: to retest the gel and stain in order to see protein expression

The samples were mixed with 9, 5 and 0 ul of water and then mixed with 10 ml of sample buffer.
Samples loaded and run at 140V setting
Gel ran about halfway down the plate.
It was left in coomassie overnight and put into the destain on the morning of the 6/24
There were no visible bands except for the die; therefore another gel should be run.

During the run time, the machine went into an alarm that said overcurrent. Attempted to change the buffer to a freshly made buffer. When this elicited the same warning as before, a different source was used. This new source was run but the samples did not move past the halfway point.

Ran Bradford on EV, and BadA

clipboard_2016-06-24_13:42:24.png

Preparing the Enzyme Assay on BadA

* Couldn't run an assay on TAT because we need taxadiene which we need fermented.

Made Ellman's Reagent

Made 10 ml of Ellman's with a 10 mM final concentration in 100% ethanol.
Added 0.00369 g of Ellman's Reagent to 10 ml of 190 proof Ethanol

Made 5 mM Benzoic Acid

Took 5 mL of 16 mM Benzoic Acid and combined with 10 ml of ultrapure water

Made 1/10 Dilution of Ellman's Reagent

Used 1 ml of Stock Ellman's Solution in 9 ml of 190 proof Ethanol

The Plate

Using a 96 well plate, each well should have:

- 10 ul of Buffer

- 25 ul of Lysate
- 10 ul of ATP
- 10 ul of Benzoic Acid (or other substrate)
- 10 ul of CoA
- 25 ul of Ellman's Reagent

Made 1/10 Dilution of Lysate

Took 20 ul of lysate and combined with 180 ul of ultra pure water.

Assay #1

Goal: To determine if we have active protein

Used a 4x4 block
The assay buffer is Mike's Buffer (contains Tris HCl)
In the wells w/o a reactant replace that volume with water

Table1

	A	B	C	D	E
1		All	w/o ATP	w/o Benzoic Acid	w/o CoA
2	EV 1				
3	EV 2				
4	BadA 1				
5	BadA 2				

All of the samples were the same shade of yellow. The results of the essay were then inconclusive. The concentrations of the Ellman's and the Lysate was then tested.

Assay #2

Goal: To Determine if different concentrations of Ellman's and Lysate Helps

Ran a 2x12 block

The assay buffer is Mike's Buffer (contains Tris HCl)
In the wells w/o a reactant replace that volume with water

Table2

	A	B	C	D	E	F	G
1		All	w/o ATP	w/o Benzoic Acid	w/o CoA	All	w/o ATP
2	EV						
3	BadA						
4		With 1/10 Ellmans	And normal lysate			with normal Ellman's	and 1/10 Ly

The shade benzoic-coA ligase (BadA) - it was found that BadA doesn't like Tris-HCl. Therefore a different buffer should be used.

Assay #3

Goal: To determine if a sodium phosphate buffer is better

A 2x4 block was used on the bottom of the same plate as Assay #2.

Table3

	A	B	C	D	E
1		All	w/o ATP	w/o Benzoic Acid	w/o CoA
2	EV				
3	BadA				

This assay was better, however, the results were still inconclusive. More research is needed into what buffer is preferable and how the paper where TJ got his numbers from did their assay.

Wk: 6/20 First Enzyme Assay

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-06-19 to 2016-06-24

FRIDAY, 6/24

Ran Gel on Proteins again

Ran another gel on the protein samples. Tested TAT, BadA, and EV. All samples had 10:0, 5:5, and 1:9 dilutions made.
Ran in new buffer; 140 V, single digit mA.
Did not run even half way down due to machine overcurrent.
Need to ask Romel what is going on!

But protein is apparently present:

Protein Gel Visualization.JPG

Wk: 6/27 Trouble Shooting BadA

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-06-27 to 2016-07-01

MONDAY, 6/27

Protein Gel Take 2

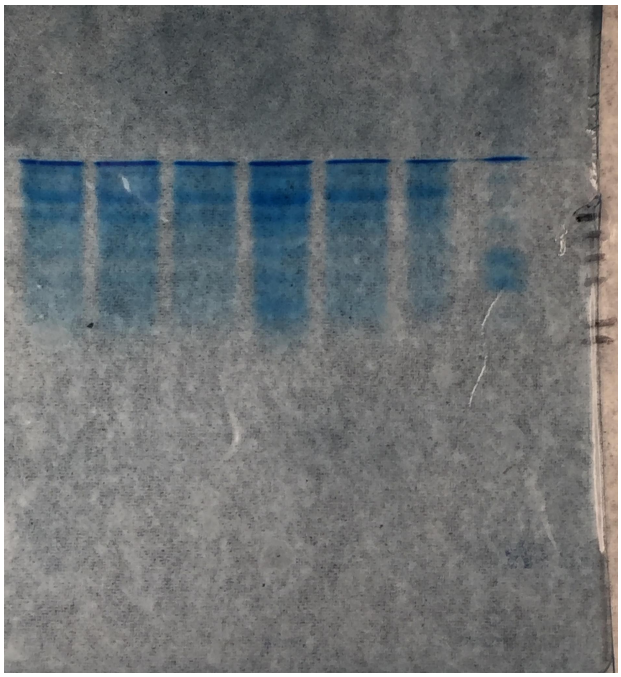
Goal: To determine if BadA is in the Lysate - Trying a different running buffer

Ran another Protein Gel Used a different Running Buffer

- 1.51 g Trisma
- 7.2 g Glycine
- 0.5 g SDS

Put into Coomassie for 1 hour. Left in destain overnight.
Also used dilutions of BadA and EV that were tailored to the protein concentrations in each
The rest of the volume up to 10 ul was filled with water
EV: 1, 2, 3
BadA: 4, 6, 8

protein gel 6:27.jpg



1: BadA 8
2: BadA 6
3: BadA 4
4: EV 3
5: EV 2
6: EV 1
7: Ladder

Wk: 6/27 Trouble Shooting BadA

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-06-27 to 2016-07-01

TUESDAY, 6/28

Buffer Screen Started

Goal: The Assays from Thursday didn't work; need to see if a different buffer helps

New Enzyme Assays need a buffer screen. Found that the enzyme may not like tris. Came up with different Buffers to try (All buffers were made in 50 mL and stored in 50 mL conical tubes in the 4 C fridge):

Table1

	A	B	C	D	E	
1	Buffer #	50 mM Na3PO4 (0.3 g)	5% Glycerol (5ml of 50%)	100 mM MgCl2 (0.5 g)	pH solution	Notes
2	1	X	X	X	KOH	A precipitate (white and cloud
3	2	X	X		KOH	100 mM MgCl2 (0.5 g) was di
4	3	X	X	X	tris base	A precipitate formed so that th
5	4	X	X	X	sodium bicarbonate	
6	5	X	X	X	tris base	Less tris was used so that the
7	6	X	X			Buffer 2 - was not pHed
8	7	X	X	X		Buffer 3 - was not pHed
9	8	X	X	X		Buffer 4 - was not pHed

Buffers Prepared for Buffer Screen

Also prepared more benzoic acid by adding 5 mL of stock benzoic acid (16 mM) to 10 mL of pH2O.

An enzyme assay was attempted but the Ellman's reagent made Thursday was determined to be expired so the results were not viable.

Protein Gel #3

Goal: To determine if the enzyme is in the insoluble fraction

Another gel was run using another Buffer:

- 50 mM MOPS
- 50 mM Tris
- 0.1% SDA
- 1 mM EDTA

The Gel was run with the empty vector, the BadA, and the insoluble fractions of BadA. The lysate and EV were run in the same dilutions as Monday. The insoluble fractions were run in dilutions of 2, 4, and 8.

The gel ran about 1/8 of the way and the machine went into overcurrent.
Put the gel in coomassie overnight:

Goal: To determine if a different coomassie/destain protocol is best

It is thought at this point that the lysate may be less pure than needed.

Wk: 6/27 Trouble Shooting BadA

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-06-27 to 2016-07-01

WEDNESDAY, 6/29

Purification of Lysate

Goal: To attempt to isolate BadA from the rest of the proteins in the lysate

Took one bottle of the BadA Lysate and centrifuged through two 100 KD filters. Took out the bottom one of the samples and set it aside. This was then labeled wash 1. Took the other and filtered through the centrifuge again. The bottom portion was then collected and set aside; it was also labeled as wash 1

Took one tube of wash 1 and filtered again through a 100 kDa filter. The top portion was then resuspended in lysate buffer and the run-through was labeled wash 1. The filtered sample from wash 1 was labeled wash 3 and the sample filtered from wash 2 was labeled wash 4.

Protein Gel #4

Goal: To Determine if the tris gel works with the tris buffer

At this point, it was confirmed that the gel we were attempting to use could only be used with a MOPS Buffer. This should have been confirmed by the third gel. We used a tris-glycine gel and ran with the tris glycine buffer from Monday.

The gel was able to run completely down the gel. The gel was then placed in Coomassie overnight.

Protein Gel #3 Cont.

The gel was transferred from the coomassie to the destain and left overnight. It was transferred around noon.

Wk: 6/27 Trouble Shooting BadA

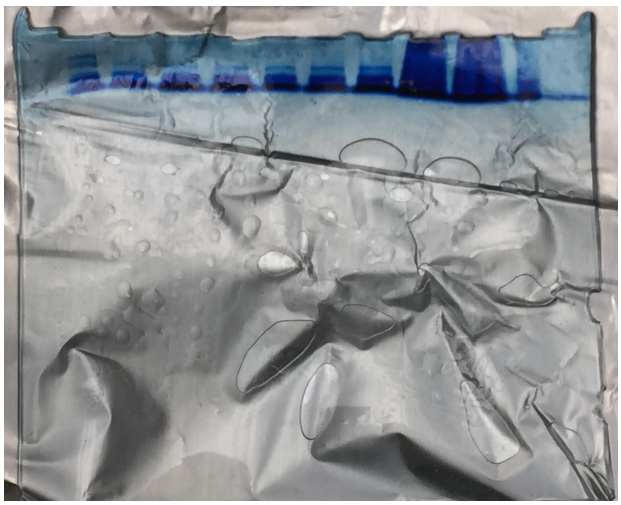
Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-06-27 to 2016-07-01

THURSDAY, 6/30

Protein Gel #3 Cont.

The Gel was taken out of the destain after 24 hours. The gel was not clear enough in the morning so it was left in and taken out after it had been in the destain for 24 hours.

6.30 Gel.jpg

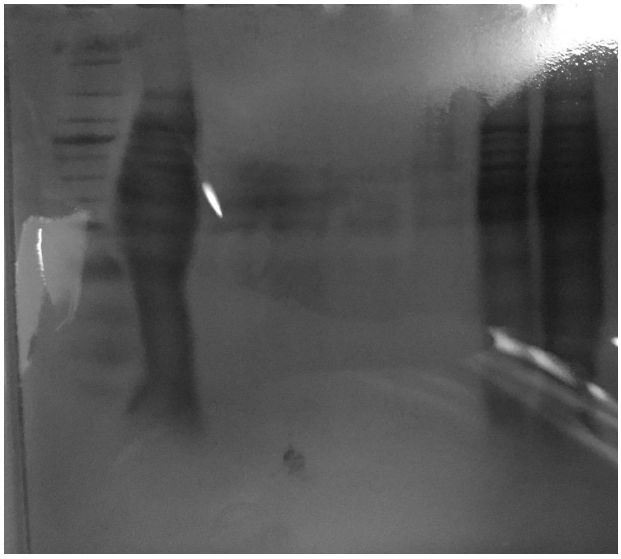


It was determined that it is feasible to leave gels in the coomassie overnight but it takes longer to destain.

Protein Gel #4 Cont.

The Gel was taken out of the coomassie in the morning and transferred to destain. The gel was allowed to sit in the destain for approximately 6 hours. At this point an analysis was taken.

IMG_1057 2.JPG



1: Ladder
2: 6 uL of Original Lysate
3: Wash 1
4: Wash 2
5: Wash 3
6: Wahs 4
7: 4 ul of Original Lysate
8: Empty Vector Original Lysate

The gel was still very blue and so the gel was left to shake in the destain overnight.

Protein Gel #5

Another gel, the 4-12% NewPAGE gel, was tested with the MOPS Buffer made on Tuesday 6/28.
It ran overcurrent very quickly and was not able to run hardly at all (less than 1 cm).

Enzyme Assays

We ran several assays. The specifications of the assays are in Table 2.

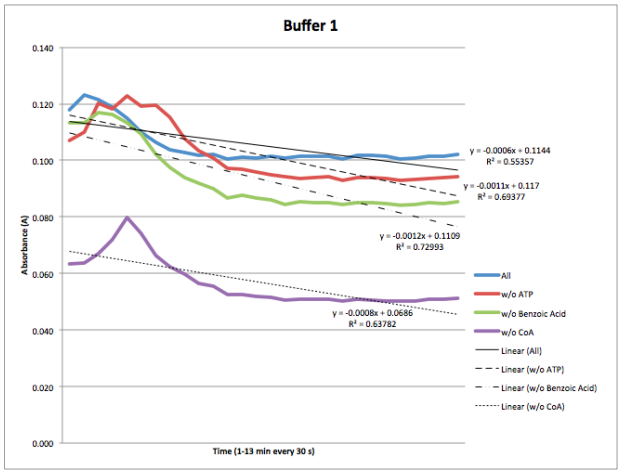
Table2

	A	B	C	D	E	F	G
1	Assay #	Sample	Columns				Buffer #
2	77	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
3	79	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
4		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
5		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
6	81	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
7		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
8		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
9	82	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
10							
11							

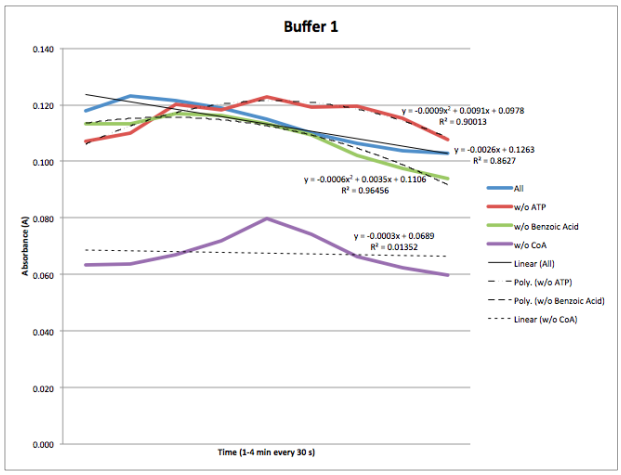
Assay #77

Incubated for 10 min at 35 C; initiated with Lysate
Used 1/10 Ellman's and 1/10 Lysate

Full Time Scale



Initial Time Period

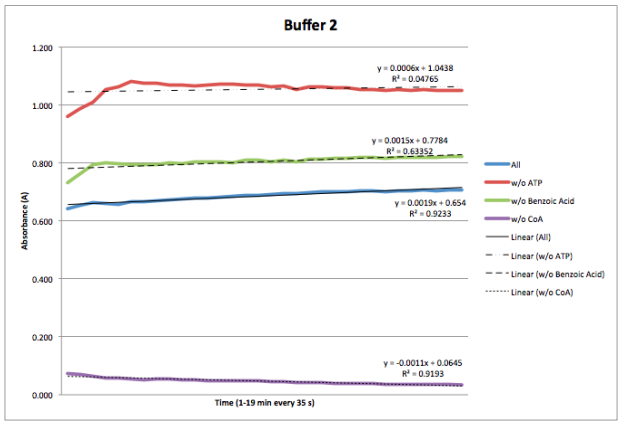


Assay #79

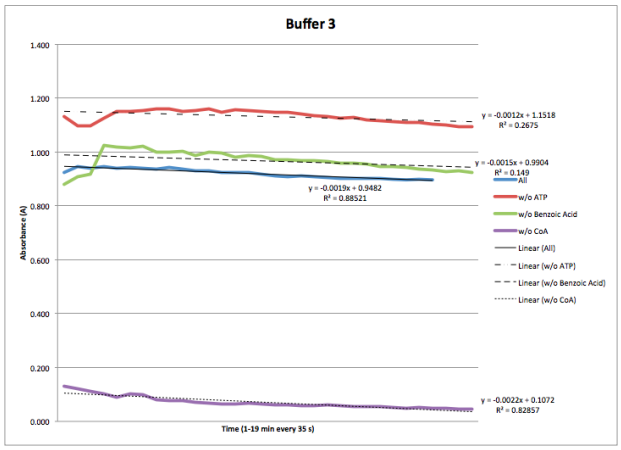
Incubated for 10 min at 35 C; initiated with Lysate

Used Regular Ellman's and 1/10 Lysate

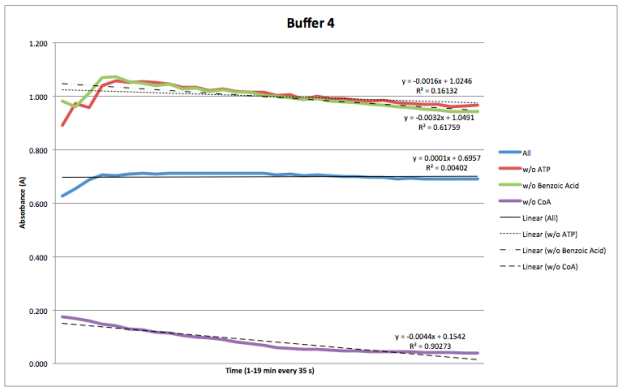
Full time scale - Buffer 2



Full Time Scale - Buffer 3



Full Time Scale - Buffer 4

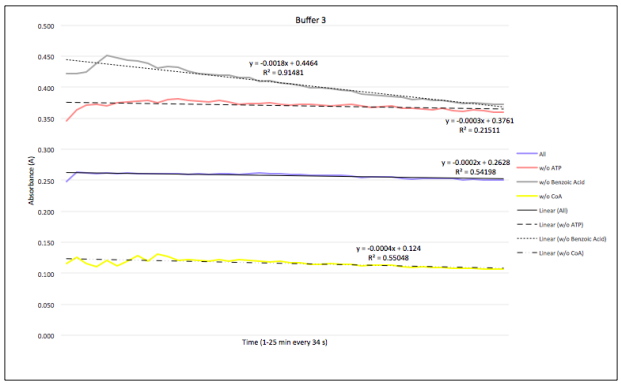


Assay #81

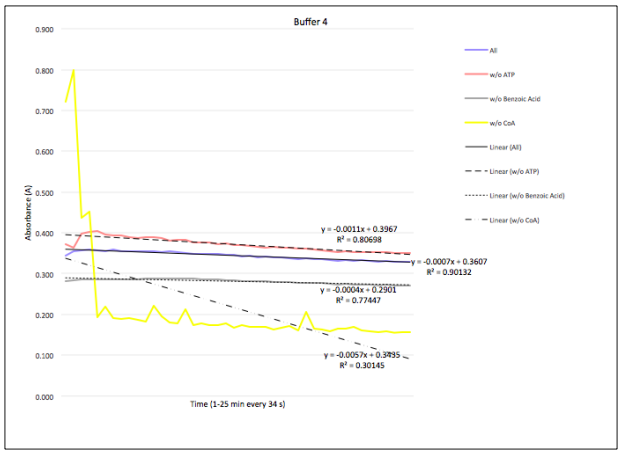
Incubated at 35 C for 10 min; initiated with Lysate

Used 1/10 lysate and 1/3 Ellman's

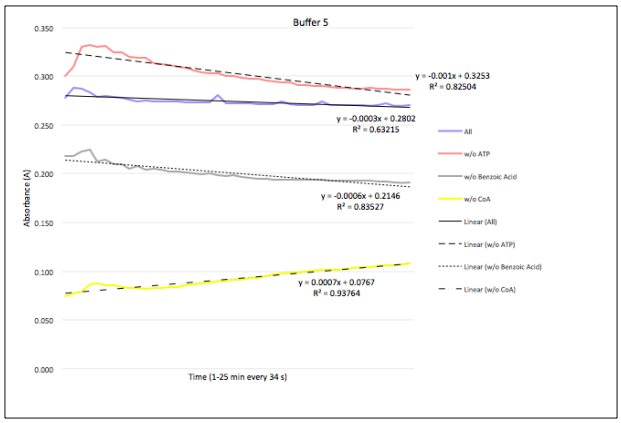
Full Time Scale - Buffer 3



Full Time Scale - Buffer 4



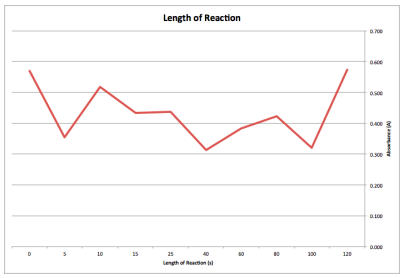
Full Time Scale - Buffer 5



Assay #82

Heated at 35 C for 10 min before placing 1/10 lysate in at various times.
Acid quenched with 1/3 ellmans after the first reaction had reacted for 2 min

Length of Reaction Trial 1



Wk: 6/27 Trouble Shooting BadA

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-06-27 to 2016-07-01

FRIDAY, 7/1

New Concentrations

After more research, new concentrations of CoA and ATP were made. 10 mM concentrations of CoA and ATP were made. Using these stocks it was then calculated that in the final volume of 90 ul;

- 2.5 uL of 10 mM ATP
- 2.5 uL of 10 mM CoA
- 10 uL of 5 mM Benzoic Acid
- 18.75 uL of Bufferr and
- 6.25 uL of MgCl2

were needed. These volumes were found by using the stock solutions and creating final concentrations in the well of 250 uM of CoA and ATP, and 100 fold concentration of 750 uM of MgCl2. This allowed for the over saturation of MgCl2 to overcompensate for the EDTA in the lysate buffer and for a 10 fold saturation of benzoic acid.

Assays

Full Ellman's was used in order to acid quench the reaction.

Wk: 7/3 BadA & DBAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-03 to 2016-07-14

SUNDAY, 7/3

To Do:

- [ ] Add the different running buffers attempted to the stock solutions page
- [ ] Update Last week with gel stuff
- [ ] Make SM10++ Media and FGM3 No P Media
- [ ] Find Protease Inhibitors (isn't this the pil thing)
- [ ] Gather data from Lab computer on Assays from Friday (only have one of the sets of data)
- [ ] Update the protocols

SM10++ Inoculation of BadA, DBAT, and Empty Vector

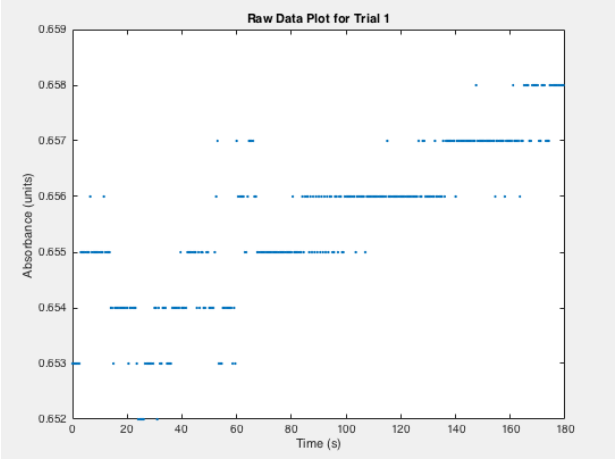
50 mL of SM10++ was placed into 4 different erlenmeyer flasks. A colony from each sample in a 286 cell was placed into each and left to grow overnight. The incubator was found (on Monday) to have remained at 29 C.

Tried two assays - Assay #9

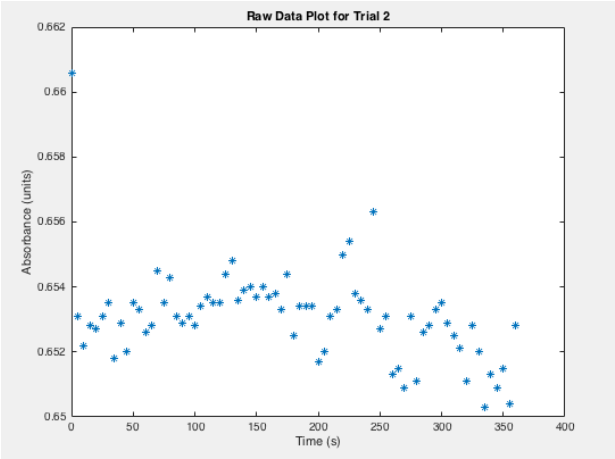
Following the Schule paper, testing of the concentration of benzoyl-CoA using 290nm was tried but taking the measurement from the spectrophotometer. This was done in a 1ml cuvette and the concentrations were adjusted. Measurements were taken first for 3 minutes using the kinetics settings and then using the photometric setting to take readings over a longer period of time. (Two trials)

- 250uM of CoA
- 5mM MgCl2
- 0.1mM of Benzoic Acid
- 250uM of ATP
- Filled to 1ml with pHed Sodium phosphate buffer

Trial 1



Trial 2


Raw Data Plot for Trial 2
Absorbance (units)
Time (s)
0.662
0.66
0.658
0.656
0.654
0.652
0.65
0
50
100
150
200
250
300
350
400

Minimal Media 2-Stage Evaluations

Introduction

This standard operating procedure describes the media formulations for preparing media for strain evaluations in various systems as well as the procedures for making concentrated stocks of each media component.

Materials

- Chemical Reagents as Mentioned in Section 1 of this SOP
- 250 mL to 1000 mL Bottles
- 250 mL to 1000 mL Flasks/Beakers
- Ultrapure Water
- 0.2 µm Filter Units, Varying Volumes
- Autoclave

Procedure

Section 1: Preparation of Stock Solutions

Prepare the stock solutions to the following concentrations using ultrapure water and ACS grade reagents.

1. Prepare 1 liter of a solution of 10X concentrated ammonium-citrate salts, varying components based on the target biomass:
2. Prepare 1 liter of **10X Ammonium-Citrate 30** salts by mixing 30 g of $(NH_4)_2SO_4$ and 1.5 g Citric Acid in water with stirring. Autoclave and store at RT.
3. Prepare 1 liter of **10X Ammonium-Citrate 90** salts by mixing 90 g of $(NH_4)_2SO_4$ and 2.5 g Citric Acid in water with stirring. Autoclave and store at RT.

Prepare the following buffering reagents:

4. **MOPS Buffer:** Prepare 1 M Potassium 3-(N-morpholino)propanesulfonic Acid (MOPS) and adjust to pH 7.4 with KOH (~40 mL). Filter sterilize (0.2 um) and store at RT.
5. **Phosphate:** Prepare 0.1 M potassium phosphate buffer, pH 6.8 by mixing 49.7 mL of 1.0 M K_2HPO_4 and 50.3 mL of 1.0 M KH_2PO_4 and adjust to a final volume of 1000 mL with ultrapure water. Filter sterilize (0.2 um) and store at RT.
6. Prepare **2 M $MgSO_4$** and **10 mM $CaSO_4$** solutions. Filter sterilize (0.2 um) and store at RT.
7. **500X Trace Metals**: Prepare a solution of micronutrients in 1000 mL of water containing 10 mL of concentrated H_2SO_4 : 0.6 g $CoSO_4$*$7H_20$, 5.0 g $CuSO_4$*$5H_20$, 0.6 g $ZnSO_4$*$7H_20$, 0.2 g Na_2MoO_4*$2H_2O$, 0.1 g H_3BO_3, and 0.3 g $MnSO_4$*H_2O. Filter sterilize (0.2 um) and store at RT in the dark.
8. **Iron Sulfate:** Prepare a fresh solution of 40 mM ferrous sulfate heptahydrate in water. Filter sterilize (0.2 um) and discard after 1 day.
9. **Thiamine:** Prepare a 50 g/L solution of thiamine-HCl. Filter sterilize (0.2 um) and store at 4°C.

10. **Glucose:** Prepare a 500 g/L solution of glucose by stirring with heat. Cool, filter sterilize (0.2 um), and store at RT.

Section 2L Final Media Compositions:

11. *Prepare the final working medium by aseptically mixing stock solutions based on the following tables in the order written to minimize precipitation.*

12. **FGM3 Growth and Production Media, pH 6.8:**

Table1

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	3.6	1.80 mM
4	Trace Metals	500 X	2	1 X
5	Fe (II) Sulfate	40 mM	2	0.08 mM
6	MgSO4	2 M	1	2.00 mM
7	CaSO4	10 mM	5	0.05 mM
8	Glucose	500 g/L	90	45 .0 g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	NA	NA
12	Casamino Acids	100 g/L	NA	NA

13. **FGM3 No Phosphate Wash and Production Media, pH 6.8:**

Table2

	A	B	C	D	E
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration	Volume in 1
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)	
3	Phosphate Buffer, pH 6.8	500 mM	0	0	
4	Trace Metals	500 X	2	1 X	
5	Fe (II) Sulfate	40 mM	2	0.08 mM	
6	MgSO4	2 M	1	2.00 mM	
7	CaSO4	10 mM	5	0.05 mM	
8	Glucose	500 g/L	50	25.0 g/L	
9	MOPS	1 M	320	200 mM	
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L	
11	Yeast Extract	100 g/L	NA	NA	#VALUE!
12	Casamino Acids	100 g/L	NA	NA	#VALUE!
13			519.8		2

14. **SM10 Seed Media, pH 6.8:**

Table3

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.0625 mM
8	Glucose	500 g/L	90	45 .0g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	10	1.0 g/L
12	Casamino Acids	100 g/L	NA	NA

15. **SM10 ++ Seed Media, pH 6.8:**

Table4

	A	B	C	D	E
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration	Volume in
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)	
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM	
4	Trace Metals	500 X	4	2 X	
5	Fe (II) Sulfate	40 mM	4	0.16 mM	
6	MgSO4	2 M	1.25	2.50 mM	
7	CaSO4	10 mM	6.25	0.06 mM	
8	Glucose	500 g/L	90	45 .0g/L	
9	MOPS	1 M	320	200 mM	
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L	
11	Yeast Extract	100 g/L	25	2.5 g/L	
12	Casamino Acids	100 g/L	25	2.5 g/L	
13	Water		414.3		2

16. **FGM10 Growth and Production Media, pH 6.8:**

Table5

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.06 mM
8	Glucose	500 g/L	50	25 .0 g/L
9	Thiamine-HCl	50 g/L	0.2	0.01 g/L
10	Yeast Extract	100 g/L	NA	NA
11	Casamino Acids	100 g/L	NA	NA

Wk: 7/3 BadA & DBAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-03 to 2016-07-14

MONDAY, 7/4

Inoculated in FGM3 - No P

Took the SM10++ and washed it. Placed it then in the FGM3 No P.

Wk: 7/3 BadA & DBAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-03 to 2016-07-14

TUESDAY, 7/5

Inoculated in SM10++

Inoculated another batch of BadA, DBAT, and EV into the SM10++. This was done in case something due to the low temperatures happened.

Made Lysate

Used the cells grown over the past two days to make more lysate. Used protocol. Made two batches; one batch was made with EDTA and protease inhibitors and one batch was made without the EDTA and protease inhibitors.

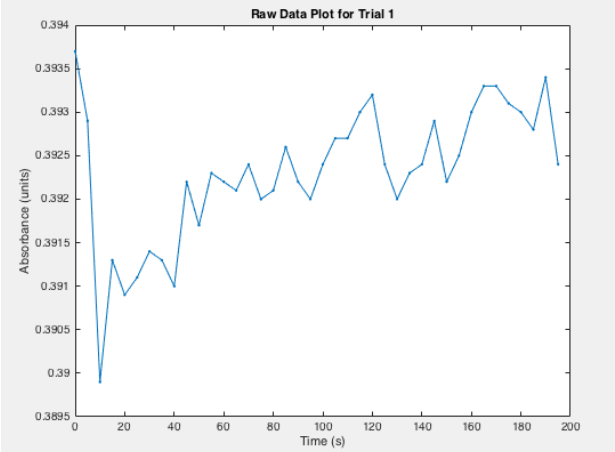
Live Cells

New Lysate was made, but ~10 mL of the fgm3 solution was set aside to do a live cell assay. The concentrations given are the final concentrations in the cuvette. A similar protocol for Assay #9 Trial 2 was used to measure the reaction.

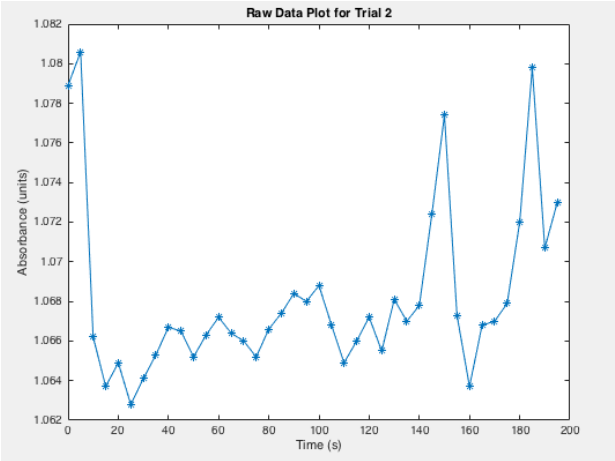
- 50 uL of 0.5 mM CoA
- 100 uL of 0.5 mM ATP
- 40 uL of 0.1 mM Benzoic Acid
- 100 uL of 5 mM MgCl2
- 500 uL of Lysate (Cells)
- 210 uL of Buffer

Two trials were conducted, one with the normal sodium phosphate buffer and the second with FGM as the buffer.

Trial 1 - Sodium Phosphate Buffer



Trial 2 - FGM Buffer



Attempted Assays

Project: Duke iGEM 2016
Authors: Thomas Luo
Date: 2016-07-06

WEDNESDAY, 7/6

#1

Used Mikes Buffer (Tris HCl- pH 7)
Recipe:

- 10 uL of Buffer
- 25 uL of Lysate
- 10 uL of ATP (50 mM)
- 10 uL of Benzoic Acid (5 mM)
- 10 uL of CoA (100 mg/mL)
- 25 uL of Ellman's (10 mM)

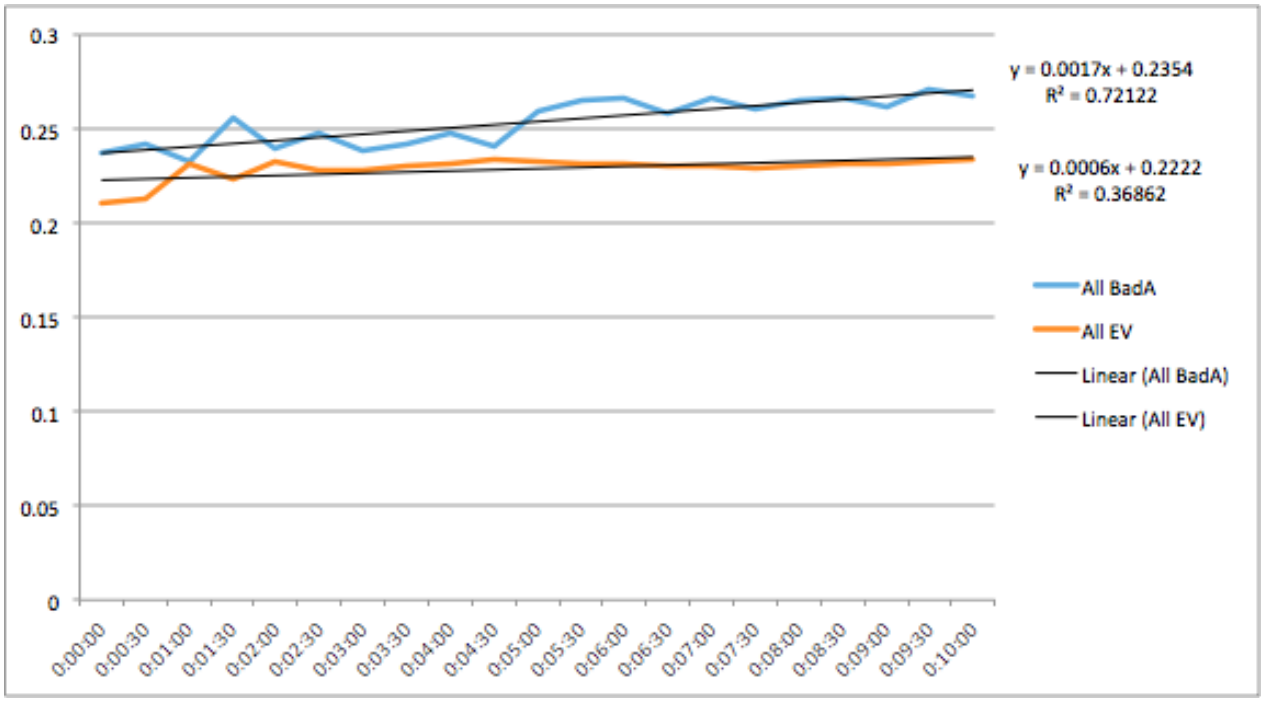
#2

Tried #1 again with different concentrations of Ellman's and Lysate
Used full concentrations, and 1/10 in three combos: full Ellman's with 1/10 Lysate, vice versa, and both 1/10 concentrations.

#3

#1 was attempted again but instead a sodium phosphate buffer was used and 1/10 concentrations of lysate and ellman's was used

Assay #3 on 6.23



Started Different Buffers

Table1

	A	B	C	D	E	
1	Buffer #	50 mM Na3PO4 (0.3 g)	5% Glycerol (5ml of 50%)	100 mM MgCl2 (0.5 g)	pH solution	Notes
2	1	X	X	X	KOH	A precipitate (white and cloudy) formed.
3	2	X	X		KOH	100 mM MgCl2 (0.5 g) was dissolved in a sep
4	3	X	X	X	tris base	A precipitate formed so that the entire solution
5	4	X	X	X	sodium bicarb.	
6	5	X	X	X	tris base	Less tris was used so that the precipitate did r
7	6	X	X			Buffer 2 - was not pHed
8	7	X	X	X		Buffer 3 - was not pHed
9	8	X	X	X		Buffer 4 - was not pHed

Assays 4-7 (Data gathered from the Teer Lab plate reader)

Table2

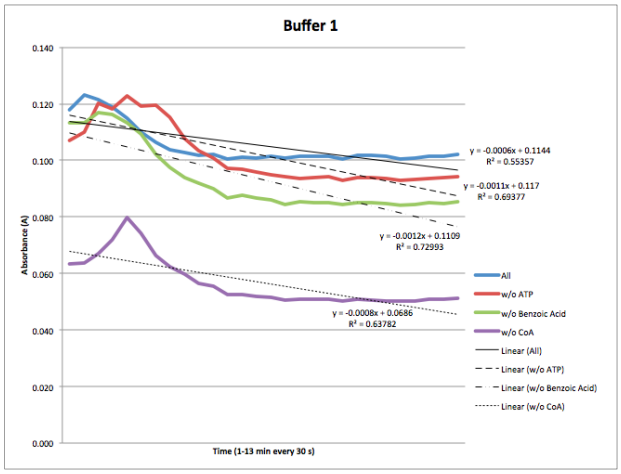
	A	B	C	D	E	F	G
1	Assay # (on machine)	Sample	Columns				Buffer #
2	77	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
3	79	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
4		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
5		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
6	81	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
7		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
8		BadA	All	w/o ATP	w/o B.A.	w/o CoA	
9	82	BadA	All	w/o ATP	w/o B.A.	w/o CoA	
10							
11							

Assay #77

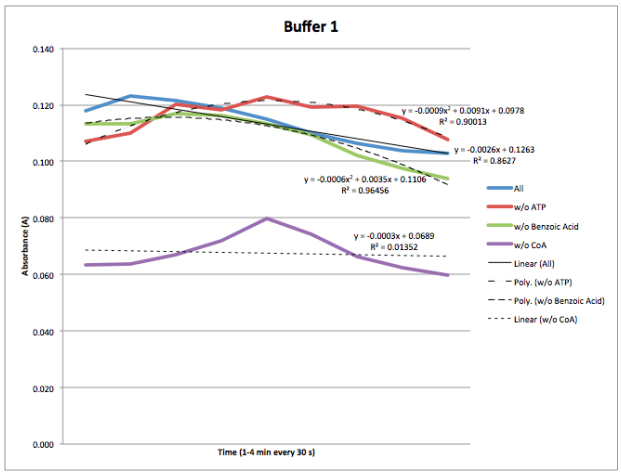
Incubated for 10 min at 35 C; initiated with Lysate

Used 1/10 Ellman's and 1/10 Lysate

Full Time Scale



Initial Time Period

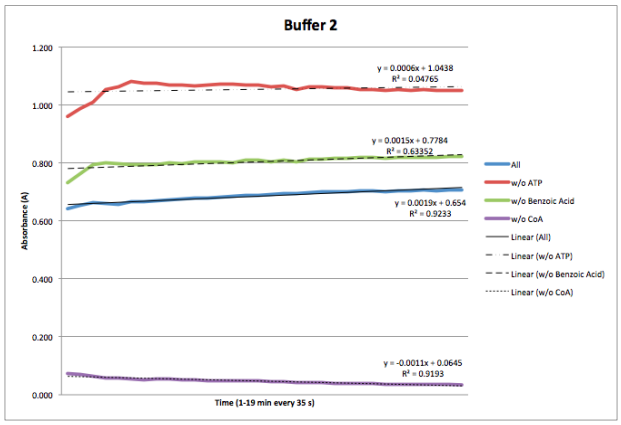


Assay #79

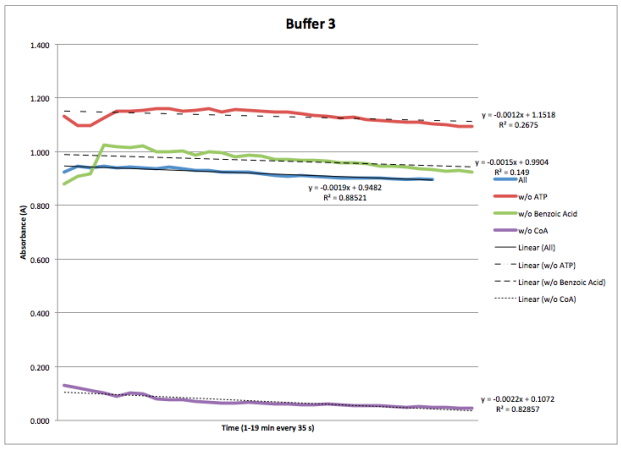
Incubated for 10 min at 35 C; initiated with Lysate

Used Regular Ellman's and 1/10 Lysate

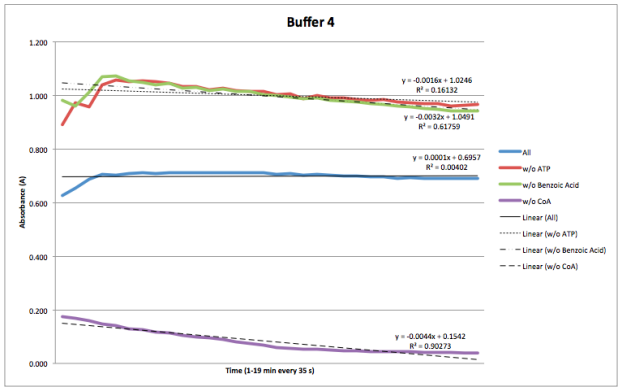
Full time scale - Buffer 2



Full Time Scale - Buffer 3



Full Time Scale - Buffer 4

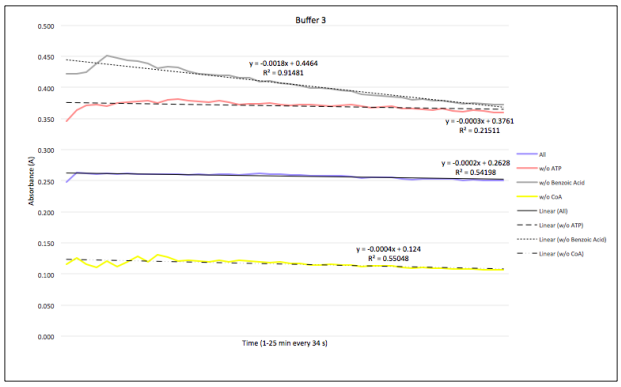


Assay #81

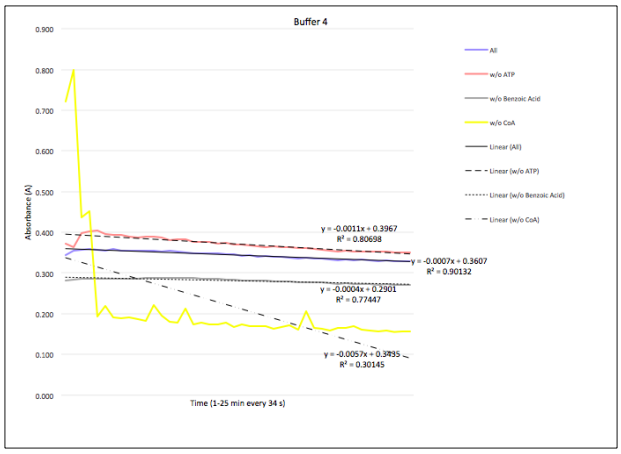
Incubated at 35 C for 10 min; initiated with Lysate

Used 1/10 lysate and 1/3 Ellman's

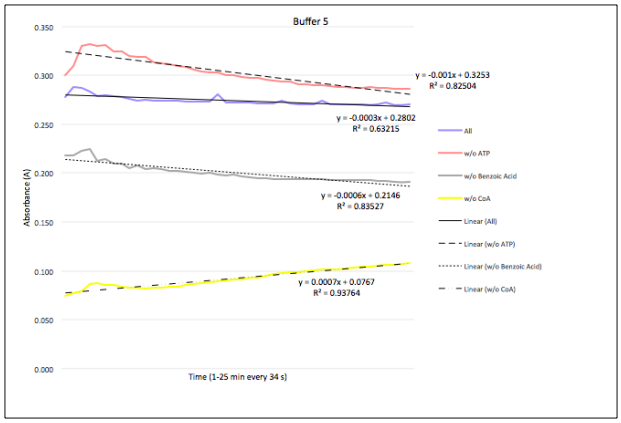
Full Time Scale - Buffer 3



Full Time Scale - Buffer 4



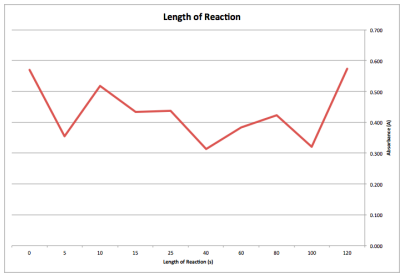
Full Time Scale - Buffer 5



Assay #82

Heated at 31 C for 10 min before placing 1/10 lysate in at various times.
Acid quenched with 1/3 ellmans after the first reaction had reacted for 2 min

Length of Reaction Trial 1



Assay #8

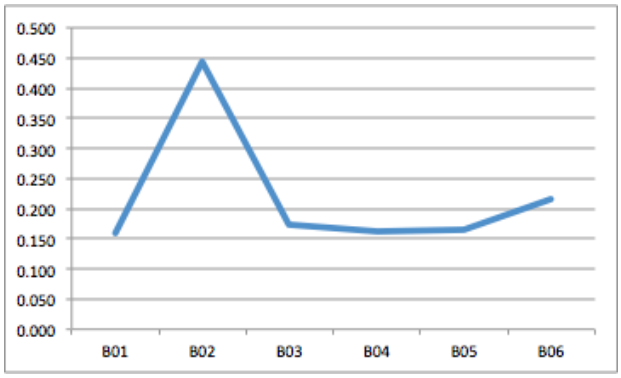
After more research (Following the Thornburg paper almost exactly), new concentrations of CoA and ATP were made. 10 mM concentrations of CoA and ATP were made. Using these stocks it was then calculated that in the final volume of 90 ul;

- 2.5 uL of 10 mM ATP
- 2.5 uL of 10 mM CoA
- 10 uL of 5 mM Benzoic Acid
- 18.75 uL of Buffer
    - Sodium phosphate buffer pHed using strips was used.

- 6.25 uL of MgCl2

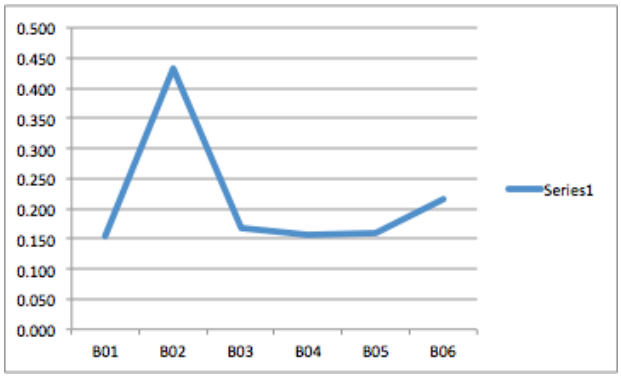
were needed. These volumes were found by using the stock solutions and creating final concentrations in the well of 250 uM of CoA and ATP, and 100 fold concentration of 750 uM of MgCl2. This allowed for the over saturation of MgCl2 to overcompensate for the EDTA in the lysate buffer and for a 10 fold saturation of benzoic acid.

Assay #91

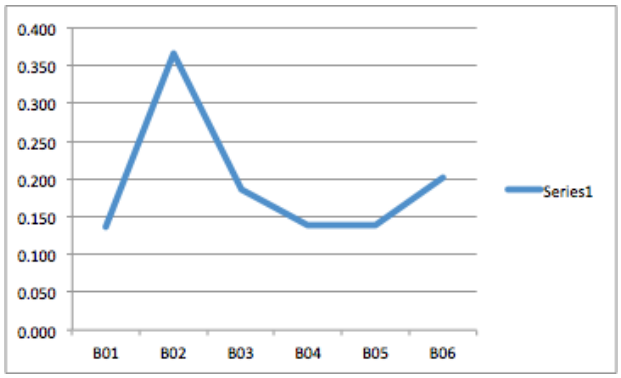


X-Axis: Time in 10 second intervals Y-axis: Absorbance at 412nm

Assay 92



Assay #93

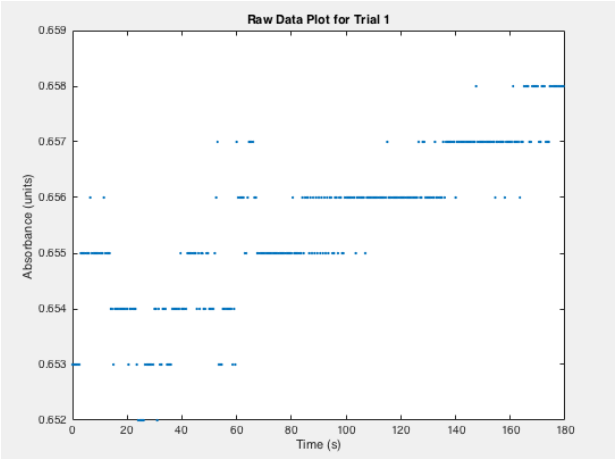


Assay #9

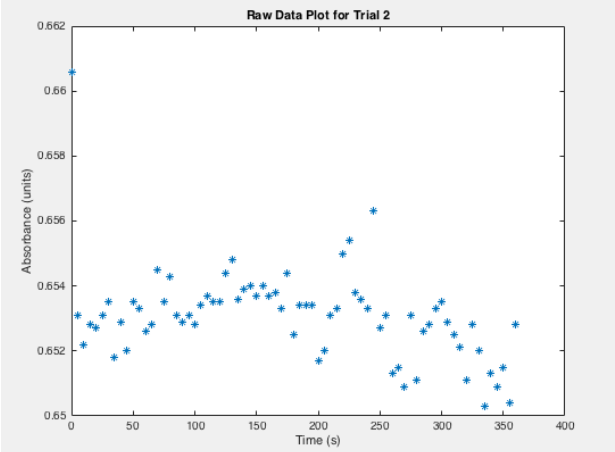
Following the Schule paper, testing of the concentration of benzoyl-CoA using 290nm was tried but taking the measurement from the spectrophotometer. This was done in a 1ml cuvette and the concentrations were adjusted. Measurements were taken first for 3 minutes using the kinetics settings and then using the photometric setting to take readings over a longer period of time. (Two trials)

- 250uM of CoA
- 5mM MgCl2
- 0.1mM of Benzoic Acid
- 250uM of ATP
- Filled to 1ml with pHed Sodium phosphate buffer

Trial 1



Trial 2



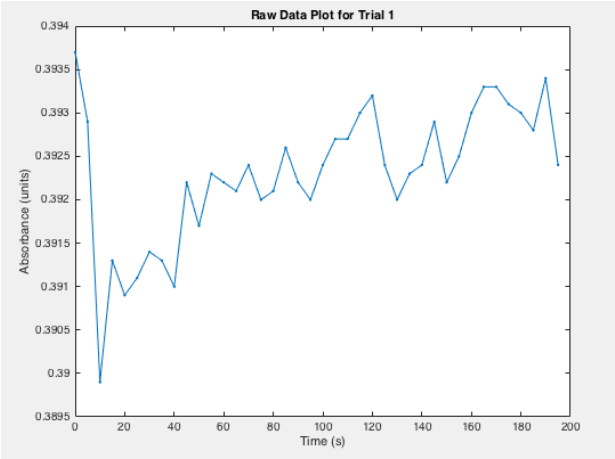
Live Cells

New Lysate was made, but ~10 mL of the fgm3 solution was set aside to do a live cell assay. The concentrations given are the final concentrations in the cuvette. A similar protocol for Assay #9 Trial 2 was used to measure the reaction.

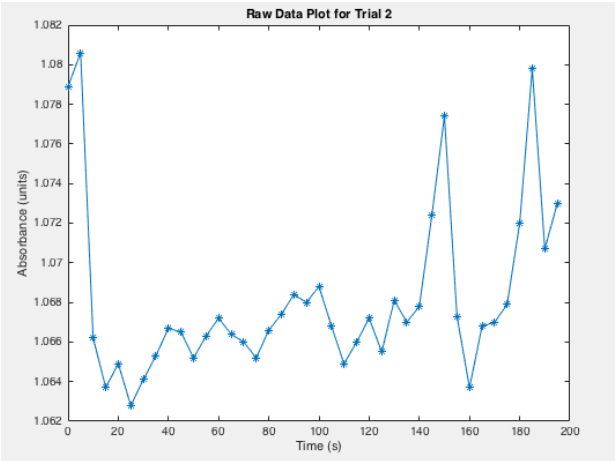
- 50 uL of 0.5 mM CoA
- 100 uL of 0.5 mM ATP
- 40 uL of 0.1 mM Benzoic Acid
- 100 uL of 5 mM MgCl2
- 500 uL of Lysate (Cells)
- 210 uL of Buffer

Two trials were conducted, one with the normal sodium phosphate buffer and the second with FGM as the buffer.

Trial 1 - Sodium Phosphate Buffer



Trial 2 - FGM Buffer



Further Experimentation

- Using 2-aminobenzoate and 355nm light detection
- Using EDTA-free lysate buffer (one paper suggested EDTA might impact enzyme)
-

Wk: 7/3 BadA & DBAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-03 to 2016-07-14

WEDNESDAY, 7/6

FGM3 - No P

The SM10++ Solution from yesterday was transferred to FGM3 No P

Wk: 7/10 Antibiotic Trials

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-10 to 2016-07-14

SUNDAY, 7/10

SM10++ Inoculation of BadA, DBAT, TAT, GFP and Empty Vector

Meeting with Mike lead us to new trial: using antibiotics and a GFP control. We were given a positive control but we needed more antibiotic before we could run.

GFP was transformed in 286 cells and allowed to grow on lb Kan plates.

Electroporation Protocol

SM10++ was made fresh. 30mg in 5ml of feSO4 was added.

We inoculated badA, DBAT, TAT GFP, EV in SM10++ with Kan added.

Minimal Media 2-Stage Evaluations

Phosphate Limited Protein Expression Protocol

Colony PCR

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

›

›

Procedure

1. Prepare econotaq master mix with oligos to amplify the region of interest (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf))

Table1

	A	B	C
1			(# col +1 control)
2	Thing	1 rxn	10
3	MM	12.5	125
4	SL1	0.25	2.5
5	SR2	0.25	2.5
6	H2O	12	120

CRITICAL COPY AND PASTE TABLE, INSERT NUMBER OF REACTIONS DESIRED AND TABLE WILL AUTO CALC VALUES

2. Aliquot out master mix + oligos into a sufficient number of PCR tubes to test colonies
3. If your backbone + insert plates have many more colonies than your backbone only or insert only, you will need to test fewer colonies than if you have lots of colonies on your control plates
4. Also include a control reaction with parent plasmid only
5. Aliquot out some LB (+ antibiotic) into another set of tubes,one for each colony you are testing
6. Pick a colony from your transformation plate with a sterile pipette tip
7. Dab the colony into the PCR tube with the econotaq master mix
8. Dab it again in the tube with LB
9. Run the PCR according to manufacturer's protocol, but with a long (5 min) initial melting step at 95-98

NOTE: When dealing with DNA samples instead of colony samples. Use 3 uL of 1ng/uL DNA

If DNA sample is poor, use at most 50 ng/uL concentration

Thermocycler Protocol

- ✔ 10. Start: 98°C for 10 min.
- ✔ 11. Cycle (x35)
- ✔ 12. Melt 98°C for 45s
- ✔ 13. Anneal 50°C for 45s
- ✔ 14. Extend 72°C for ___
- ✔ 15. Time = (1 min / kbase)*length(longest amplificant)
- ✔ 16. 4°C for infinity

Wk: 7/10 Antibiotic Trials

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-10 to 2016-07-14

MONDAY, 7/11

FgM3NoP Inoculation of BadA, DBAT, and Empty Vector

Minimal Media 2-Stage Evaluations

FgM3NoP was made fresh - 500ml.

cells for extracting RNA samples were taken from the SM10++ by mixing 1 OD of cells with 0.5ml of RNAlater.

Samples were centrifuged. washed in fg3noP 20ml.

Resuspended in fg3noP+kan.

Wk: 7/10 Antibiotic Trials

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-10 to 2016-07-14

TUESDAY, 7/12

limited phosphate cells taken out after 20 hours.

Sample of cells was taken for RNA preservtion
GFP cells were placed under UV light - no green coloring was observed

SDS Page Protocol (Protein Gel)

Protein gel was run:

cells were mixed with sample buffer and heated at 98 degrees for 30 minutes
sample was spun down and the supernatant was loaded.

Gel ran for 1:45 minutes

Wk: 7/10 Antibiotic Trials

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-10 to 2016-07-14

WEDNESDAY, 7/13

Drag and drop a file from the sidebar to attach or

UPLOAD A FILE

Wk: 7/10 Antibiotic Trials

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-10 to 2016-07-14

THURSDAY, 7/14

Colony PCR

Colony PCR Ingredients

	A	B	C
1			(# col +1 control)
2	Thing	1 rxn	16
3	MM	12.5	200
4	SL1	0.25	4
5	SR2	0.25	4
6	H2O	12	192

File_000.jpeg

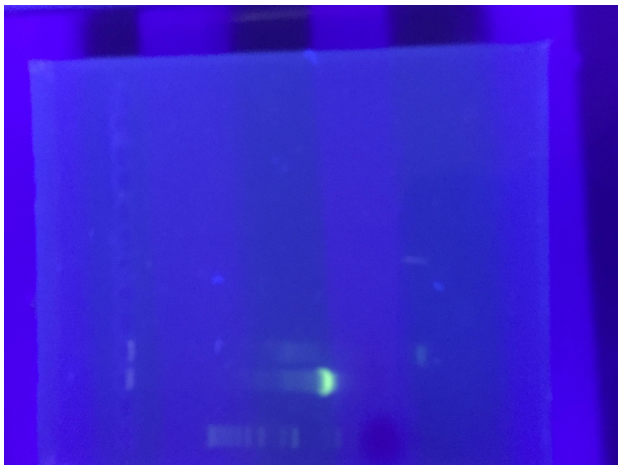
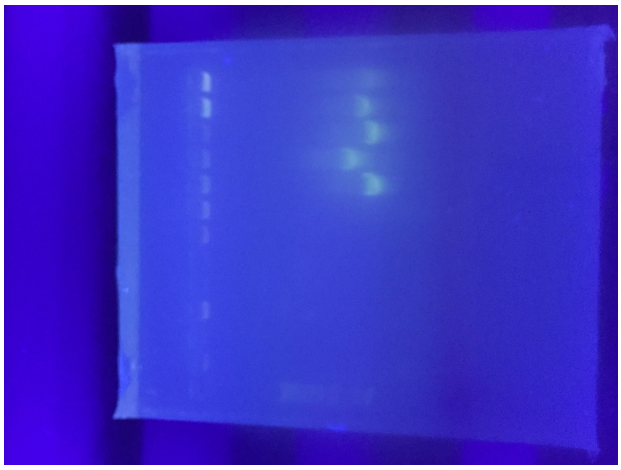


Table1

	A	B
1	1	L
2	2	GFP
3	3	PSM

File_000.jpeg



Bottom is the ladder = well 1

Table2

	A	B	C
1	1	L	
2	2	GFPA	Frozen
3	3	EV-D	F
4	4	DBAT	F
5	5	TAT	F
6	6	BADA	F
7	7	EV-B	F
8	8	GFPB	F
9	9	DBAT	
10	10	EV-D	
11	11	PAM	
12	12	TAT	
13	13	BADA	
14	14	TBT	

Wk: 7/3 BadA & DBAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-03 to 2016-07-14

THURSDAY, 7/14

The Samples grown over the last two days were spun down and frozen but no lysate or test was done because it was concluded that the process hadn't changed enough to have protein expressed due to the lack of antibiotic.

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

FRIDAY, 7/15

SM10++ and FGM3 were made. Special care was taken to ensure sterility. Both medias were filter sterilized into sterile bottles once all ingredients had been mixed.

New Iron Sulfate was made. An error in previous calculations was found. It was determined that in 5 mL 0.556 g of Iron sulfate was needed.

It is worth noting that the FGM3 turned a more yellow/green color instead of clear (this is a good indication that the recipe is better than before as the media should be yellow). New MOPS buffer was used for the FGM.

Both medias were left on the benchtop to check for contamination. After two days, nothing had grown in the media.

Colony PCR

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

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›

Procedure

1. Prepare econotaq master mix with oligos to amplify the region of interest (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf))

Table1

	A	B	C	D
1			(# col +1 control)	
2	Thing	1 rxn	28	
3	MM	12.5	350	
4	SL1	7.815	218.82	Using 3.2 uM
5	SR2	0.25	7	
6	H2O	4.435	124.18	

CRITICAL COPY AND PASTE TABLE, INSERT NUMBER OF REACTIONS DESIRED AND TABLE WILL AUTO CALC VALUES

2. Aliquot out master mix + oligos into a sufficient number of PCR tubes to test colonies
3. If your backbone + insert plates have many more colonies than your backbone only or insert only, you will need to test fewer colonies than if you have lots of colonies on your control plates
4. Also include a control reaction with parent plasmid only
5. Aliquot out some LB (+ antibiotic) into another set of tubes,one for each colony you are testing
6. Pick a colony from your transformation plate with a sterile pipette tip
7. Dab the colony into the PCR tube with the econotaq master mix
8. Dab it again in the tube with LB
9. Run the PCR according to manufacturer's protocol, but with a long (5 min) initial melting step at 95-98

NOTE: When dealing with DNA samples instead of colony samples. Use 3 uL of 1ng/uL DNA

If DNA sample is poor, use at most 50 ng/uL concentration

Thermocycler Protocol

- ✔ 10. Start: 98°C for 10 min.
- ✔ 11. Cycle (x35)
- ✔ 12. Melt 98°C for 45s
- ✔ 13. Anneal 50°C for 45s
- ✔ 14. Extend 72°C for ___
- ✔ 15. Time = (1 min / kbase)*length(longest amplificant)
- ✔ 16. 4°C for infinity

Lysate Buffer

Introduction

Storage of

Materials

›

›

Procedure

1. Phosphate buffer 2mM
2. EDTA (200ul)
3. Protease inhibiotrs (0.5 pill)
4. water to dilute
5. Made in a 50 ml conical tube and stored in 4 C fridge.

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

SUNDAY, 7/17

In the morning a starter culture of samples was begun. Samples were taken from plates (marked with a green marker) and inoculated in ~3 mL of SM10++ & KAN. An sample of the media with no inoculation was also taken and showed no growth after 8 hours. After 8 hours the starter cultures were added to 50 mL of the same media in flat flasks. The flasks were incubated at 37 C and shaken at ~200 rpm for 19 hours.

Samples:

- DBAT
- BadA
- PAM
- TAT
- EV T
- EV B
- EV D
- GFP A
- GFP B

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

MONDAY, 7/18

After the samples had grown for 16 hours, the OD at 600nm was checked using a spectrophotometer. The OD was ~4. After the samples had been incubated for 19 hours, the OD was checked again. The OD was ~7.

Samples were taken for RNA and plates. For RNA samples 250ul of sample was mixed with 2ml of RNAlater and cooled in the fridger for 1 and half hours before being moved to the -20 freezer. 25 ul of each sample was plated onto a plain LB plate and 25 ul of each samples was plated onto an LB + KAN. The plates were grown overnight and the results can be seen below.

INSERT PHOTOS HERE

Once all samples were plated and had RNA samples made of them, they were spun down for 20 min at 4000 rpm at 4 C. This was done in the Buchler Lab. After this first spin down, the supernatant was discarded. Samples were resuspended in 25 ml of FGM3-No P. The samples were then spun down again at the same rpm/time. This supernatant was discarded again. Samples were then resuspended in 50 ml of FGM3 No P + KAN and placed into flat flasks. The flasks were grown in a shaker at ~200 rpm for **~20** hours at 37 C.

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

TUESDAY, 7/19

After 18 hours, the GFP controls were placed onto the UV plate along with an empty vector control. The GFP controls turned glowing green and the empty vector did not. This was a good indication that the medias worked. The samples were left in the shaker for another couple hours.

INSERT PHOTO HERE

Before the samples were spun down, samples were taken for RNA (of all) and plates (PAM, TAT, DBAT, BADA) using the same protocol as above.

Following the protocol given by rommel/mike the cells were spun down at 4k rpm for 20min and then placed directly into the freezer.

A protein Gel was run on the samples. Samples for the gel were first taken from stock samples (50 uL) and spun down. The samples were then resuspended in lysate buffer with EDTA (10 ul) and 10 uL of SDS Sample Buffer

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

WEDNESDAY, 7/20

Several assays were attempted on lysate. Half of the lysate of BadA, EV, and GFP. Assays were conducted with different temperatures, concentrations, ect. Due to results of the protein gel, the lysate did not have any enzyme, none of the enzymes worked.

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

THURSDAY, 7/21

Using lysate from the day before, another assay was attempted using a longer viewing period. However, because of the lack of enzyme, this assay was also negative.

Wk: 7/17 Expression of BadA, DBAT, PAM, and TAT

Project: Duke iGEM 2016
Authors: evmiles
Dates: 2016-07-15 to 2016-07-22

FRIDAY, 7/22

The other half of the lysate was filtered again differently. This time it was filtered with a 10 kD filter instead of a 30 kD filter. This was in an effort to see if the enzyme was being filtered out accidentally.

Wk: 7/25 Final Attempts

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-07-25 to 2016-07-28

MONDAY, 7/25

Over the weekend, more assays were attempted. All of them were negative as there was no enzyme in the lysate. Another batch of SM10++ was started.

Today, the SM10++ was changed with a wash to FGM NoP. Another batch of SM10++ was started.

Spec matb cells were created as a positive control because they have been demonstrated to work previously

Code was also written to perform the Michaelis Menten calculations.

Wk: 7/25 Final Attempts

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-07-25 to 2016-07-28

TUESDAY, 7/26

The FGM3 batch from Monday was spun down and some samples were lysed. The SM10++ was changed with a wash to FGM3 NoP
Another batch of SM10++ was started

Assays were attempted with the BadA samples. Live cell assay - 10 different concentrations were attempted. The live cell assay seemed to work but ultimately failed.

Another protein gel was run. The gel was negative

Wk: 7/25 Final Attempts

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-07-25 to 2016-07-28

WEDNESDAY, 7/27

The SM10++ from the day before was changed to FGM3 No P

The FGM3 was spun down and stored.

Another gel was run on the cells from the FGM3 no P

Wk: 7/25 Final Attempts

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-07-25 to 2016-07-28

THURSDAY, 7/28

The Fgm3 noP from yesterday was dumped out. An assay was attempted with Malonic Acid. The assay failed. Some of the samples from the FGM3 were lysed for the assay. Another protein gel was run. Again it was negative.

Troubleshooting happened. See troubleshooting entry for details.

Troubleshooting Notes End of August

Project: Duke iGEM 2016
Authors: evmiles
Date: 2016-07-29

FRIDAY, 7/29

The process of growing cells includes steps:

- Inoculating cells from the plate
- Taking the inoculation and adding it to a flat flask
- Taking the grown cells (OD600 of >=10) and wash once in FGM3 NoP
- Taking the washed cells and adding to a flask of FGM3 NoP
- Allowing the cells to express for about 24 hours
- Taking the cells, spinning them down
- Either storing the cells at -80 or lysing them immediately

After spending time in the FGM3 No P the media has historically turned red. This is thought to be incorrect at this point. The color of the media can change; usually it is due to enzyme being released into the media and the enzyme is colored. This would be normal. However, each enzyme should, theoretically, express differently and have a different color. Because all of our enzymes as well as our empty vector control (which shouldn't have any color and is the most important evidence for this argument) are turning red, the red color is thought to be contamination. Theoretically, even if one of the enzymes produces something that is red, the other enzymes should be different colors and the GFP positive control should be green, not green mixed with red.

In order to control for contamination, there are several procedures we have already been practicing. The first is when making the media. The pico pure water that we collect from the Lynch lab and keep in the jug that is next to the sink is not sterile water. The water in the jug has been deionized and somewhat sterilized by a UV light, however, it is still not sterile. When making the media, it is important that we make the media in a beaker or Erlenmeyer flask and then filter sterilize the media into a sterile bottle. Leave the media out on the counter and be sure to toss it out after a week (it is therefor important for you to date the media when you make it. The second is when aliquoting the media into flasks and culture tubes. When aliquoting the media, be sure to ethanol the bottle, hands, flasks, bench etc. Unless the serological pipette is coming out of a sterile wrapping, that should be sterilized with ethanol and dried in the air thoroughly before use. Be sure to be extremely careful when attempting sterile technique. This is a very good place in the process for contamination to be introduced.

There are several places that contamination can be introduced. even using the practices above. The first is the cells that we are using. The 286 plates that were used throughout the month of July were taken from the 286 plate that was contaminated. While care was taken to chose a colony that was far away from any contamination, it is very possible that contamination still occurred. A second place where contamination could have been introduced is during the wash process. Because the Teer Lab does not have a large centrifuge, the wash has always taken place in another lab. This means that generally, there is not a proper bench that we using. It would be easy at this point, for an over full cell waste (which is easy to have once you have emptied 10-12 samples twice) to splash back to the tube, for contamination on a piece of equipment to touch all of your samples, etc. Therefore, from this point forward, there are two options. The first option to address this problem is to not do a wash. The purpose of the wash is to wash away any extra phosphate left over. This is not strictly necessary but is generally a good idea. If a wash doesn't occur, then any phosphate that remains in the media will just be used up by the cells and once it is all gone, the cells will express the gene. If this course of action is adopted, it is important that the cells are spun down for a full 20 minutes at 4,000 rpm. The second course of action is to proceed with the wash, but do so under sterile conditions. This would entail going to another lab to spin the samples down (15 or 20 minutes should be fine) and then bringing the samples back to the Teer Lab. At this point, the bottles, gloves, etc. should be sterilized with ethanol. Then the samples should be emptied of the SM10++ media, and 25 mL of FGM3 No P added. Then the samples should be resuspended and taken back to the other lab to get spun down again. The samples can then be brought back to the Teer Lab. Once back, the samples can be dispensed of their supernatant and then added to the flat flask after resuspension. If this option is preferred, it is very important that sterile technique is used at all times.

In general, to reduce risk of contamination, there are several policies that should be put into practice. Before handling different samples hands should be cleaned with ethanol. This will prevent one sample from contaminating the other samples.

In order to solve our problem with the 286 cells, we have started a new line of the 286 cells. We took comp cells from the Lynch lab and made new plates with them. Comp cells have yet to be made with the new line. All old comp cells of 286 should be tossed. In order to completely change the cell line, mini-prepped DNA should be transformed into the new line and all old plates disregarded. This hopefully can happen during August so that protein characterization can immediately resume come September. Before August, the cells were used to transform Empty Vector to see if there was a difference in structure between the Lynch Cells and Teer Cells. This experiment did not result in any growth. It is therefore important that a few bottles of the old 286 Comp Cells are kept in order to redo the experiment to see if the hypothesis of contamination is correct.

Wk: 9/4 List & Schedule

Project: Duke iGEM 2016
Authors: Nisakorn Valyasevi
Date: 2016-09-03

SATURDAY, 9/3

emma reminder

- add protocols to to do list
- write optical density protocol
- add protocol for rna samples

To Do:

- [ ] Colony PCR of BadA and DBAT that are in new 286
  - [ ] Jay performed on 9/3 - visualizing Mon 9/5 due to lack of stain
- [ ] Transform PAM, TAT, TBT, Spec, GFP and BAPT into new 286
  - [ ] Use "Rom 8/31" and/or "Jen 8/31" comp cells
  - [ ] Transform two samples of each
  - [ ] When plating, plate 75 uL on a plate
  - [ ] Take extra care when spreading
  - [ ] Use very sterile technique
  - [ ] Take care to use recovery media QUICKLY! Be as fast as possible while still being sterile!
- [ ] Colony PCR PAM TAT TBT Spec GFP and BAPT that are in new 286
  - [ ] Happens after successful transformations above
- [ ] Express BadA DBAT GFP SPEC EV

  - [ ] Colony PCR from plates collected between each step
    - [ ] after SM10 and FGM take 5 uL of the solution (shake well before taking the sample) and mix in an epi tube 95 uL of the media (SM10 or FGM)
    - [ ] Then plate the solution.
    - [ ] After all samples have been taken, a PCR needs to happen to ensure there is plasmid in our cells
  - [ ] Take RNA Samples this week
    - [ ] After SM10 and FGM take a specific amount and make RNA samples **Thomas edit protocol for what needs to happen and what you want**
      - [ ] mix 1ml per 1 OD
  - [ ] Starter cultures must be done 12 hours before samples in SM10 are desired
  - [ ] 50 mL of SM10 for 24 hours
  - [ ] Wash with FGM (10-15 mL)
  - [ ] 50 mL of FGM for 24 hours
  - [ ] spin down and then freeze in -80 freezer

  - [ ] Be sure to be very sterile! Sterilize all equipment before and after.
  - [ ] Do not touch tubes twice in a row with the same equipment without sterilizing the equipment
  - [ ] Ethanol and dry hands if ANY possibility exists that liquid fell onto your hands
- [ ] Sonicate Samples
  - [ ] Follow sonication protocol
  - [ ] **Thomas! How do we want these labeled and stored?**
    - [ ] These samples can be stored in the -80 for protein gels, but shouldnt be stored for enzyme assay since every freeze-thaw cycle reduces the enzyme activity (badA doesn't have much to begin with)
  - [ ] Thes samples sholdn't be stored for enzyme assay but
- [ ] Run Gels
  - [ ] Run gels on the live cells and on lysate
  - [ ] See Thomas or Emma - do not attempt alone
- [ ] Run Assays

- [ ] Run assays on live cells and on lysate on the spec and on plate reader
- [ ] See Thomas or Emma - do not attempt alone

Stocks:

- [ ] Make running buffer
  - [ ] Parth? - need **Thomas what recipe are we using?**
  - [ ] 5X Running buffer 500ml: 7.55g Trisbase, 36g glycine, 2.5g sds
- [ ] Make SM10 and FGM
  - [ ] BE EXTREMELY STERILE!
  - [ ] Make 500 mL of each
  - [ ] Use the 500 mL sterile filter bottles (bottle is already attached to the filter)
- [ ] Make stock of MOPS
  - [ ] Make a liter of MOPS - pH adjust in lynch lab with KOH (under fume hood)

Admin

- [ ] Need stain for Colony PCR
- [ ] Buy nickle for histidine tags
- [ ] Ordering more MOPS
- [ ] Plan protocol for RNAc
- [ ] Check for supplies for the RNAc

Table1

	A	B	C	D	E	F	G
1	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2	Planning	Sm10 and fgm made	Sm10	Fgm Wash	Spin down	Sonicate	
3	Colony PCR on plates in fridge	Make MOPS & Running Buffer					Colony PC the plates f each step
4							
5		Colony PCR cont.				Assays	
6		Transformations & Colony PCR on Transformations				Gels	Gels Cont.
7							
8		Visualize Colony PCR from 9/3 - Nisa AM					
9	Start SM10 Starter Cultures - Thomas/Nisa/Jay/Gina/ect.	SM10 Transfer AM	FGM Wash & Transfer 9 am Emma	Spin Down and Freeze - Nisa			
10			Starter Cultures at Night - Emma	SM10 started - Nisa	FGM Wash - Thomas	Live Cell Gels & Assay - Emma	
11		Transformations & Colony PCR on Transformations					
12		Make Running Buffer; MOPS; SM10 & FGM - Parth/Nisa	Transformations Parth & Emma	Colony PCR on the transformations - Adam	Colony PCR on the transformations - Adam		

Monday!

SM10++ Transfer

Take the starter cultures from last night and add to SM10 in a flat flask. Total volume within the flask is 50 mL. This is performing DAY 1 of the protocol below. Be sure to use sterile technique! Don't use the same serilogical pipette on two samples, ethanol hands frequently, etc.

Phosphate Limited Protein Expression Protocol

Visualize Colony PCR

Made FGM no Phosphate

Minimal Media 2-Stage Evaluations

Introduction

This standard operating procedure describes the media formulations for preparing media for strain evaluations in various systems as well as the procedures for making concentrated stocks of each media component.

Materials

- Chemical Reagents as Mentioned in Section 1 of this SOP
- 250 mL to 1000 mL Bottles
- 250 mL to 1000 mL Flasks/Beakers
- Ultrapure Water
- 0.2 µm Filter Units, Varying Volumes
- Autoclave

Procedure

Section 1: Preparation of Stock Solutions

Prepare the stock solutions to the following concentrations using ultrapure water and ACS grade reagents.

1. Prepare 1 liter of a solution of 10X concentrated ammonium-citrate salts, varying components based on the target biomass:
2. Prepare 1 liter of **10X Ammonium-Citrate 30** salts by mixing 30 g of $(NH_4)_2SO_4$ and 1.5 g Citric Acid in water with stirring. Autoclave and store at RT.
3. Prepare 1 liter of **10X Ammonium-Citrate 90** salts by mixing 90 g of $(NH_4)_2SO_4$ and 2.5 g Citric Acid in water with stirring. Autoclave and store at RT.

Prepare the following buffering reagents:

4. **MOPS Buffer:** Prepare 1 M Potassium 3-(N-morpholino)propanesulfonic Acid (MOPS) and adjust to pH 7.4 with KOH (~40 mL). Filter sterilize (0.2 um) and store at RT.
5. **Phosphate:** Prepare 0.1 M potassium phosphate buffer, pH 6.8 by mixing 49.7 mL of 1.0 M K_2HPO_4 and 50.3 mL of 1.0 M KH_2PO_4 and adjust to a final volume of 1000 mL with ultrapure water. Filter sterilize (0.2 um) and store at RT.
6. Prepare **2 M $MgSO_4$** and **10 mM $CaSO_4$** solutions. Filter sterilize (0.2 um) and store at RT.
7. **500X Trace Metals**: Prepare a solution of micronutrients in 1000 mL of water containing 10 mL of concentrated H_2SO_4 : 0.6 g $CoSO_4$*$7H_20$, 5.0 g $CuSO_4$*$5H_20$, 0.6 g $ZnSO_4$*$7H_20$, 0.2 g Na_2MoO_4*$2H_2O$, 0.1 g H_3BO_3, and 0.3 g $MnSO_4$*H_2O. Filter sterilize (0.2 um) and store at RT in the dark.
8. **Iron Sulfate:** Prepare a fresh solution of 40 mM ferrous sulfate heptahydrate in water. Filter sterilize (0.2 um) and discard after 1 day.
9. **Thiamine:** Prepare a 50 g/L solution of thiamine-HCl. Filter sterilize (0.2 um) and store at 4°C.

10. **Glucose:** Prepare a 500 g/L solution of glucose by stirring with heat. Cool, filter sterilize (0.2 um), and store at RT.

Section 2L Final Media Compositions:

11. *Prepare the final working medium by aseptically mixing stock solutions based on the following tables in the order written to minimize precipitation.*

12. **FGM3 Growth and Production Media, pH 6.8:**

Table1

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	3.6	1.80 mM
4	Trace Metals	500 X	2	1 X
5	Fe (II) Sulfate	40 mM	2	0.08 mM
6	MgSO4	2 M	1	2.00 mM
7	CaSO4	10 mM	5	0.05 mM
8	Glucose	500 g/L	90	45 .0 g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	NA	NA
12	Casamino Acids	100 g/L	NA	NA

13. **FGM3 No Phosphate Wash and Production Media, pH 6.8:**

Table2

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 30 Salts, pH 7.5	10 X (30 g Ammonium Sulfate, 1.5 g Citrate)	100	1 X (3 g Ammonium Sulfate, 0.15 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	0	0
4	Trace Metals	500 X	2	1 X
5	Fe (II) Sulfate	40 mM	2	0.08 mM
6	MgSO4	2 M	1	2.00 mM
7	CaSO4	10 mM	5	0.05 mM
8	Glucose	500 g/L	50	25.0 g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	NA	NA
12	Casamino Acids	100 g/L	NA	NA

14. **SM10 Seed Media, pH 6.8:**

Table3

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.0625 mM
8	Glucose	500 g/L	90	45 .0g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	10	1.0 g/L
12	Casamino Acids	100 g/L	NA	NA

15. **SM10 ++ Seed Media, pH 6.8:**

Table4

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.06 mM
8	Glucose	500 g/L	90	45 .0g/L
9	MOPS	1 M	200	200 mM
10	Thiamine-HCl	50 g/L	0.2	0.01 g/L
11	Yeast Extract	100 g/L	25	2.5 g/L
12	Casamino Acids	100 g/L	25	2.5 g/L

16. **FGM10 Growth and Production Media, pH 6.8:**

Table5

	A	B	C	D
1	Ingredient	Concentration Stock	Volume in 1 L (mL)	Final Concentration
2	Ammonium-Citrate 90 Salts, pH 7.5	10 X (90 g Ammonium Sulfate, 2.5 g Citrate)	100	1 X (9 g Ammonium Sulfate, 0.25 g Citrate)
3	Phosphate Buffer, pH 6.8	500 mM	10	5.00 mM
4	Trace Metals	500 X	4	2 X
5	Fe (II) Sulfate	40 mM	4	0.16 mM
6	MgSO4	2 M	1.25	2.50 mM
7	CaSO4	10 mM	6.25	0.06 mM
8	Glucose	500 g/L	50	25 .0 g/L
9	Thiamine-HCl	50 g/L	0.2	0.01 g/L
10	Yeast Extract	100 g/L	NA	NA
11	Casamino Acids	100 g/L	NA	NA

Phosphate Limited Protein Expression Protocol

Introduction

This standard operating procedure (SOP) describes a protocol for the evaluation of heterologous enzyme expression which is induced by low phosphate conditions.

There are two phases when expressing proteins: (i) growth and (ii) expression. In the first phase, we inoculate media and allow cells to grow and reproduce to reach target cell densities. Once the cells are growing exponentially, we induce for protein production (expression phase).

Protein expression works by activating the promoter associated with your genes of interest (eg. enzyme). There are many different promoters that activate through different signals. In this SOP we use promoters that get activated under low phosphate concentrations. Thus, to induce protein expression (activate a low phosphate inducible promoter), we exchange the media to a media that lacks phosphate.

Once induction has occurred, time is needed for transcription and translation. For protein expression using our system, we evaluate protein expression after at least 16 hours.

When harvesting the cells to assay for protein expression, we centrifuge them and resuspend in a lower volume. This will result in a higher final protein concentration and easier-to-handle volumes.

To extract the proteins (eg enzymes), one needs to lyse the cells. Again, there are many methods available, but we will use sonication. Sonication involves introducing a fine metal tip that vibrates at a high speed. This vibration causes the formation of air bubbles that grow until they explode, creating waves that disrupt cell integrity. Important to note is that sonication causes a lot of heat, so cells need to be lysed in ice. In addition, many cells naturally contain proteases which may degrade your protein, activity of proteases is reduced on ice during lysis. In addition, we may add protease inhibitors to further reduce potential protein degradation.

To confirm production of proteins, one can use sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, a sample of cell lysate is taken and heated briefly at 95 C in the presence of a free thiol reducing agent such as beta-mercaptoethanol or dithiothreitol (DTT) and sodium dodecyl sulfate (SDS). Heat will denature proteins, beta-mercaptoethanol will break disulfide bonds in the protein and SDS which is negatively charged will stick to the denatured proteins and give the proteins an associated number of negative charges based which is based in the size or molecular weight of the protein. Then this mixture of denatured proteins can be run in a polyacrylamide gel exposed to a voltage causing negatively charged species to run through the gel, and since negative charges are added proportionally to the proteins size while bigger proteins get hung up in the gel or run slowere, SDS-PAGE separates proteins based on size. This gel can then be stained to detect protein bands on it. Overexpressed proteins will appear as larger bands.

Materials

- Growth Media: SM10+ Media, pH 6.8, has excess phosphate (1L) –See Appendices Below
- Production Media: SM10-noP Media, pH 6.8 (1L) –See Appendices Below
- Microcentrifuge Tubes
- Disposable Cuvettes
- 10 mL conical centrifuge tubes
- 50 mL conical centrifuge tubes
- 1 mL microcentrifuge tubes
- Spectrophotometer
- Temperature Adjustable Shaking Incubator set to desired temperature (usually 37C)
- 250-mL Non-baffled Shake Flasks
- Antibiotics

- Media Supplements/Induction Chemicals (Thiamine, Anhydrotetracycline, etc.)
- Protease Inhibitor Cocktail (if used)
- Ice bucket and ice
- Sonicator
- SDS-PAGE Components - See SDS Page protocol

Procedure

Day 1:

1. 1. Using Colonies inoculate one new 50 mL culture of each strain to be evaluated in SM10++ defined medium with antibiotics or supplements as required in square culture flasks. Incubate 16 hours in an incubator shaker at 220 rpm and 37 C. Be sure to start an empty vector control cultire.

Day 2:

2. Measure the OD600 of the overnight cultures and record.

   When measuring OD, make sure the values you are getting are between 0 and 0.5. Depending in the particular spectrophotometer, anything above that is not within the linear measuring range of the apparatus and, therefore, is not a reliable measurement. To make sure you are in the linear range, do a 1:10-20 dilution of your culture before measuring the OD. Put this diluted sample in a spectrophotometer cuvette. Set the spectrophotometer to measure absorbance at 600 nm. Fill one cuvette with 1 ml of sterile media or water and blank the spectrophotometer. Then, measure the OD of your sample.

3. Once the cells have reached an OD of 4-6, proceed with below steps.
4. Transfer cultures to 50 mL conical tubes and centrifuge at 4,000 RPM for 20 minutes.
5. Resuspend the cells in 50 mL of FGM3-noP defined medium with antibiotics or supplements as required in square culture flasks. Incubate for hours to allow for protein expression.

Day 3:

6. Transfer cultures to 50 mL conical tubes and centrifuge at 4,000 RPM for 20 minutes.

   This is a good stopping point. You can put the cells in the -80 C freezer. Do not freeze cells that have already been resuspended.

7. Resuspend the cells in 2-5 mL of 50 mM Phosphate buffer pH 8.0 with protease inhibitors and 2 mM EDTA (be mindful that these protease inhibitors or EDTA may inhibit the enzyme you are trying to obtain, so do not add them if that is the case). Transfer the cells in 10 mL conical tubes.

   Best to use 3 ml.
   The lysate buffer can be premade in the lysate buffer protocol.

8. Place the 10 mL conical tubes on ice and sonicate for 3 minutes, 10s-on/30s-off cycles, at 50% tip amplitude (DO NOT increase the amplitude beyond 50%, this is tip specific)
9. Centrifuge down the cell debris at 10,000 rpm at 4 C for 10-15 minutes.
10. Take out and reserve the cell lysate as it should contain your protein of interest (if it is in the soluble fractions, sometime your protein may be expressed but be insoluble so may be spun down or insoluble fraction for analysis as well). Put the cell lysate in 500 uL aliquots in 1 mL microcentrifuge tubes

11. Carefully remove the supernatant into a 30 kDa filter tube

    These are found under where the DNA concentration machine is

12. Spin at 6.7k for 5-10 minutes R

13. Repeat three times adding 200-800ul of lysis buffer or the appropriate buffer for the enzyme assay

    Each time, pipette up and down to prevent the filter from getting clogged.

14. After the last spin, keep the concentrated lower volume lysate.

    The top part will have more liquid than the flow through. This is what is supposed to happen.

15. Take the final liquid on the top and aliqout (800ul) into epi tubes.

16. Add (534 ul) of 50% glycerol to each aliquot

17. Store in -80 C freezer.

18. The cell debris should have twice as much volume of cell debris of 50% glycerol added to it and place into the -80 C freezer.

    We will run a sample of the cell debris on a SDS-PAGE to make sure your protein is not in the insoluble fraction of the cell lysate.

Wk: 9/4 Work

Project: Duke iGEM 2016
Authors: Parth Patel
Dates: 2016-09-04 to 2016-09-09

SUNDAY, 9/4

Update each day with notes (be very specific and descriptive) of what you did when you did it (include times) include any notes on sterility. Also include volume notations. Also include pictures!!!! I know its annoying but its important that others can see colors of things, where things are located specifically ect. Also notate where items are left.

After you are done each day it is vital the lab is picked up! Last week it was left messy several times!!!

Thomas/Nisa/Jay

1. Colony PCR samples moved to 4 degrees
2. Starter cultures were made in lb+kan: EV-R, EV-J, BADA-B, BADA-Q5, DBAT

Wk: 9/4 Work

Project: Duke iGEM 2016
Authors: Parth Patel
Dates: 2016-09-04 to 2016-09-09

MONDAY, 9/5

Thomas/Nisa/Gina

Starter cultures added to flat flasks

- Thomas and Nisa added the 3ml starter cultures to 47ml of sm10++
- placed in shaker at 225rpm

FGM-3 NoP was made:

- New Iron sulfate was made 56mg in 5ml of water and then placed into the fridge
- Fgm was the correct green color
- fgm was vaccum filtered in hood
- Kan was added after filteration. (500ul)

Minimal Media 2-Stage Evaluations

Gel electrophoresis

Parth (3:20- 6:40 pm)

Running Buffer

- 5X Running buffer 1L: 15.1g Trisbase, 72g glycine, 5g sds
- pH adjusted from 5.4 to 7.5 (added 3 mL of kOH)

Result: Precipitation overtime, likely culprit is the Tris-HCl being used instead of Trisbase

Wk: 9/4 Work

Project: Duke iGEM 2016
Authors: Parth Patel
Dates: 2016-09-04 to 2016-09-09

TUESDAY, 9/6

Emma

Transformed 2 samples of each TBT, TAT, and PAM - placed in incubator - good numbers just of low values.

Should be attempted Again.

Checked the OD of SM10 - 6.38 at 10:00 am - left in incubator until afternoon

Plated transformations after a 2 hour recovery - 75 ul on each plate

FGM Wash - Parth and Jay

Wk: 9/4 Work

Project: Duke iGEM 2016
Authors: Parth Patel
Dates: 2016-09-04 to 2016-09-09

FRIDAY, 9/9

Parth, Thomas, Emma (1:25- ___ pm)

Plated FGM samples

- DBAT-3
- EV-J1-3

Remade 5x Running Buffer

-

Wk: 9/11 List and Schedule

Project: Duke iGEM 2016
Authors: evmiles
Date: 2016-09-11

SUNDAY, 9/11

Last Week

Mike Transformed Tax10 and TycA
Assay Worked ish

Monday:

Make:

- [x] FGM - Nisa
- [x] SM10 - Nisa
    - Ran out of MOPS fore making FGM, so had to put SM10 on hold
- [x] MOPS - Nisa (be careful)
- [x] KAN Plates - Nisa
    - Poured 24 normal, 20 abnormal denoted by star (the Kan potentially degraded due to wait time at a high temp)
- [x] Inoculations for Jay - Parth

Autoclave:

Note: Always bring the heat gloves from our lab with you to autoclave and do not leave in autoclave room

- some ass took the designated heat gloves from the autoclave room

- [ ] Trash - Parth
    - (Not enough trash)
- [x] Bottles - Parth
- [x] Glassware - Parth
- [x] Pipette Tips (already in the Bin) - Parth

To Do:

- Colony PCR on SM10 plates (in fridge txt emma if you need help) - Adam (Wednesday)
    - We don't have access to the PCR machine on Tuesday
    - same primers different plate - labelled SM10 in fridge
- Mini Prep TAT TBT BAPT(need AMP) BadA - Jay (Tuesday)
    - BAPT is not really in the sequence confirmed box

Tuesday:

Sonicate - Emma (1:30 - 5)

Parth Compels You To:

- [ ] Talk to Cranky Lady and ask her where the damned plates are
    - [ ] If we have no plates, go to Lynch Lab and tell Adim to order you some
- [ ] Make sure more culture tubes for innoculation are avaliable
- [ ] Make sure I used the right plates for the inoculations (Jaydeep has info I sent)
- [ ] Test the plates I poured for Nisa after I was trying to find the goddamned plates and make sure only Kan resistant things grow
- [ ] (Optional) Prepare some LB+Amp into small aliquots

5 on Assays
Gel

Ben can prepare box to take with beforehand
Bradford

Tuesday pm

sm10 starter cultures -Jay

SM10++ + KAN Starter Cultures (5 mL) put into shaker at 12:30 PM for DBAT, BadA, GFP, EV

Minipreps done for BAPT in LCAmp (23 ng/uL), BadA (115 ng/uL), TAT (98 ng/uL), TBT (116 ng/uL) > Put into sequence confirmed box, top left.

Wednesday AM

SM10 started - nisa

Thursday

FGM Wash - ben

- [ ] Spun (4K@20min) down samples (286-GFPA, EV-R2, DBAT 100uL, BadA-O5 100uL),
- [ ] Dumped liquid, resuspended in 25mL FGM+Kan
- [ ] Spun again (4K@20min). Discarded
- [ ] Resuspended in 50mL FGM (+Kan added)
- [ ] Placed in shaker at 2pm

Friday

Gels and assays after 3

- Took 50 mL samples and spun down for 20 min@4K
- Sonicated Samples for 3 min 10s on/30s off
- Filtered samples using filtration protocol
- Ran the SDS page
  - Used 1 uL of samples as well as 5 uL of samples
  - Incubated for ~5 min
  - Ran gel for ~2 hours and 10 min
    - Would not run farther and so pulled gel
  - Put gel in comassie overnight
- Ran Assay on BadA
  - 25 uL ATP
  - 18.75 uL of Buffer
  - 6.25 uL of MgCl2
  - 2.5 uL of CoA
  - 25 uL of Ellmans
  - vaired amounts of Benzoic Acid

  - Left overnight
  -

Antibiotic Stocks

Introduction

Three kinds of antibiotic stocks (1000x). These recipes make 1000X stocks that follow the recommendations for plates.

Materials

- Antibiotic Powder
  - Stored in -20°C freezer
- Pico Water

Procedure

Kanamycin Stocks (10 mL)

1. Use 10 mL of pico water (autoclaved dH2O)
2. Add 400 mg of Kanamycin powder (-20°C freezer)
3. Mix well
4. Filter [using antibiotic filters and syringes (in drawer underneath middle bench)] into aliquots of 1 ml epi tubes
5. Store aliquots labeled with concentration (1000x), date, and KAN in -20°C freezer

Ampicillin Stock (10 mL)

6. Use 10 mL of pico water (autoclaved dH2O)
7. Add 1 g of Ampicillin powder (-20°C freezer)
8. Mix well
9. Filter using antibiotic filters into 1 mL aliquots (epi tubes)
10. Store aliquots labeled with concentration (1000x), date, and AMP in -20°C freezer

Chloramphenicol Stock (10 mL)

11. Use 10 mL of ethanol
12. Add 250 mg of Chloramphenicol powder (-20°C freezer)
13. Mix well
14. Filter using antibiotic filters into 1 mL aliquots (epi tubes)
15. Store aliquots labeled with concentration (1000x), date, and CM in -20°C freezer

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

SATURDAY, 9/17

Parth (6:00-6:30)

Made Inoculations of PSMART-LC TycA

- used -80°C freezer stock
- created two 5 mL culture tubes with LB+Kan

Colony PCR

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

›

›

Procedure

1. Prepare econotaq master mix with oligos to amplify the region of interest (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf (https://www.lucigen.com/docs/manuals/MA038-EconoTaq-PLUS.pdf))

Table1

	A	B	C
1			(# col +1 control)
2	Thing	1 rxn	10
3	MM	12.5	125
4	SL1	0.25	2.5
5	SR2	0.25	2.5
6	H2O	12	120

CRITICAL COPY AND PASTE TABLE, INSERT NUMBER OF REACTIONS DESIRED AND TABLE WILL AUTO CALC VALUES

2. Aliquot out master mix + oligos into a sufficient number of PCR tubes to test colonies
3. If your backbone + insert plates have many more colonies than your backbone only or insert only, you will need to test fewer colonies than if you have lots of colonies on your control plates
4. Also include a control reaction with parent plasmid only
5. Aliquot out some LB (+ antibiotic) into another set of tubes,one for each colony you are testing
6. Pick a colony from your transformation plate with a sterile pipette tip
7. Dab the colony into the PCR tube with the econotaq master mix
8. Dab it again in the tube with LB
9. Run the PCR according to manufacturer's protocol, but with a long (5 min) initial melting step at 95-98

NOTE: When dealing with DNA samples instead of colony samples. Use 3 uL of 1ng/uL DNA

If DNA sample is poor, use at most 50 ng/uL concentration

Thermocycler Protocol

- ✔ 10. Start: 98°C for 10 min.
- ✔ 11. Cycle (x35)
- ✔ 12. Melt 98°C for 45s
- ✔ 13. Anneal 50°C for 45s
- ✔ 14. Extend 72°C for ___
- ✔ 15. Time = (1 min / kbase)*length(longest amplificant)
- ✔ 16. 4°C for infinity

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

SUNDAY, 9/18

- [ ] Transform TAX10 and TycA into 286 - Parth Monday PM
- [ ] TycA Sequence Confirmed - Adam
- [ ] Email the mini cystron
- [x] Make SM10++ (no antibiotic)
- [x] Make FGM (no antibiotic)

Parth -
Monday after 6
Wednesday Afternoon
Thursday Afternoon

Nisa -
Monday till 4
Wednesday till 4

Jay -
Tuesday 2-5
thursday 10-3
Friday after 3

Adam -

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

SUNDAY, 9/18

Parth (5:00-6:30)

Miniprep of PSMART-LC TycA

- used the NEB Miniprep Kit
- Concentrations were 33ng/uL and 59 ng/uL
- Stored in the -20°C , sequence confirmed box, bottom left corner

Mike's List

Project: Duke iGEM 2016
Authors: evmiles
Date: 2016-09-19

MONDAY, 9/19

1) GFP Expression Report - blue
    a) Time course of floursecence after wash : GFP vs. EV
    b) SDS PAGE Gel showing GFP induction : GFP vs. EV
2) Improved iGEM vector report - Orange
    a) Failed Gibson Trials with iGEM vector sequences - include #s and sequence of failed clones, including tax10
    b) iGEM vector Repair with Q5 Mutagenesis
    c) successful gibson with new overhangs in pSMART (tax10)
    d) successful gibson with new overhangs in iGEM backbone (tax10)
3) Replace RBS for badA, DBAT, BAPT - green
    a) Q5 mutagenesis
    b) get MDL primer sequences ASAP to order
4) Protein Expression (ideally 3 parts SDS Page, enzyme assay, analytical confirmation)
    a) tycA, tax10 first try protocol and SDS PAGE again, can do western if needed
5) Anaytical Methods (Mike will help)
    a) Taxol
    b) benzoyl-CoA
    c) baccatin III
    d) phenyl-isoserine-CoA
    e) N-debenzoyl-taxol
    f) benzoic aci
    g) phenylisoserine
    h) 10-deactyl baccatin
6) In-vitro taxol production by mixing lysates
    a) express all five genes, make lysates, mix and look for taxol in combined enzyme assay
6) Golden Gate Assembly GGA of 5 genes - light blue
7) In-vivo production of taxol with GGA vector

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

MONDAY, 9/19

SM10++ and FGM made (Nisa)

- came in 11:30, finished 12:30
- filtered at 2:30
- made 1 L of each
- stored in 4 degrees by Thomas

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

MONDAY, 9/19

Parth (6:30-9:00)

Electroporation and Transformations of PSMART-LC TycA, TAX10

Table1

	A	B	C
1	TycA (1st try)	320 mV	654 ms
2	TycA (2nd try)	1660 mV	2.8 ms
3	TAX10	1660 mV	2.8 ms

Plated on 5 plates (two are being tested to see whether they have the proper antibiotic)

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

TUESDAY, 9/20

Put together the mini cistron

AM: Start SM10++ Cultures of

- BadA
- DBAT
- TycA
- Tax10
- BAPT

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

TUESDAY, 9/20

Jaydeep (6:30 - 9:30 PM)

2 x Electroporation and Transformation of PSmart-LCKan TycA & TAX10
Rommel's 286 Cells used for Transformations
1 uL DNA: 35 uL Cells

Table2

	A	B	C
1	TAX10-5 (1)	1630 V	2.7 ms
2	TAX10-5 (2)	1640 V	2.9 ms
3	TycA 78 ng/uL (1)	1640 ms	2.8 ms
4	TycA 78 ng/uL (2)	1660 V	3.0 ms

Recovered for 1 hour, 25 min.
10 & 100 uL of each transformation plated 9:25 PM

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

WEDNESDAY, 9/21

Noon: FGM Wash
Needs 24 hours to incubate

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

WEDNESDAY, 9/21

Jaydeep (2:45 - 3:45 PM)

Colony PCR of 9/20 Plates

Table3

	A	B	C
1			(# col +1 control)
2	Thing	1 rxn	13.2
3	EconoTaq MM-2X	12.5	165
4	SL1 (100 uM)	0.25	3.3
5	SR2 (100 uM)	0.25	3.3
6	H2O	12	158.4

12 reactions: TBT-M, TBT-C, TAX10 (1-5), TycA (1-5)

4:45 elongation time, old PCR machine, finshed at 7:45 PM and left overnight

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

THURSDAY, 9/22

AM: Sonication

PM: Assay

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

THURSDAY, 9/22

Jaydeep (9:45 AM - 12:45 PM)

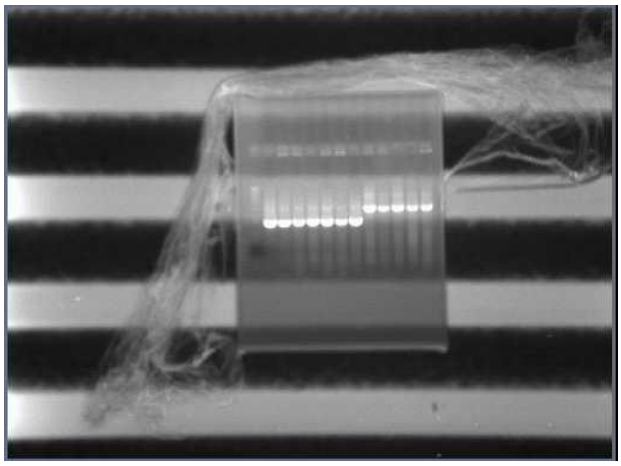
Gel Screening of PCR Products

- 53 ml TAE 1x
- 0.53 g agarose
- 5.3 uL DNA gel stain (MIDORI Green)
- Reused TAE 0.5x buffer
- Ran for 25 min. @ 160 V

Table4

	A	B	C	D	E	F
1	Well	1	2	3	4-8	9-13
2	Substance	Ladder	TBT-M	TBT-C	TAX10 (1-5)	TycA (1-5)

TAX10TycA092216Refocus.jpg



TAX10 and TycA Positive
Note that ladder was run slightly late, so DNA is slightly longer than it appears relative to ladder in the picture
TAX10 and TycA plates are on near-bottom shelf in 4 degree fridge, tested colonies marked in green; all plates screened here should be dated 9/20

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

FRIDAY, 9/23

AM: Running a Gel

Emma

Run a Gel with Mike to determine what is going wrong with running our gels

3 PM: Mike & Mass Spec

All

Mike will go over how to perform analytical tests on Assay results using the mass spectrometer

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

FRIDAY, 9/23

Jaydeep & Thomas (5 - 6 PM)

6 starter cultures in 3 mL SM10++KAN: TAX10, TycA, DBAT, BadA, EV, GFP
1 starter culture in 3 mL SM10++AMP: BAPT

Autoclaved trash

Wk: 9/18 List and Schedule

Project: Duke iGEM 2016
Authors: Thomas Luo
Dates: 2016-09-18 to 2016-09-24

SATURDAY, 9/24

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

SATURDAY, 9/24

Jaydeep (10 - 11 AM)

6 starter cultures grew and were combined with analgous 47 mL of media in flat flasks: TAX10, TycA, DBAT. BadA, EV, BAPT
1 starter culture did not grow and the plate was heavily inoculated straight into 50 mL SM10++KAN in a flat flask: GFP

Wk: 9/18 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-17 to 2016-09-25

SUNDAY, 9/25

Jaydeep & Thomas (11 AM - 1:30 PM)

Black specks observed in spun down BAPT, also different color in FGM-noP flask - Thomas thinks possible contamination
FGM wash of cultures, 50 uL of antibiotic added to 25 mL FGM-noP in each flask before adding 25 mL FGM-noP resuspended, washed cells to each flask

Put in incubator at 1:30 PM

Wk: 9/25 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-27 to 2016-09-28

TUESDAY, 9/27

Jaydeep (7 - 11 PM)

Q5 PCRs on BadA for RBS replacement

- Hydrated 2 primers to 100 uM and made 10 uM stocks in oligos box
- 63 degrees Celsius annealing, 2:00 elongation
- 3 PCRs: on original DNA @ 100 ng/ul as well as 1:10 and 1:100 dilutions

Q5 PCRs on BAPT for RBS replacement

- Hydrated 2 primers to 100 uM and made 10 uM stocks in oligos box
- 62 degrees Celsius annealing, 2:00 elongation
- 3 PCRs: on original DNA @ 23 ng/uL as well as 1:10 and 1:20 dilutions

pSB1C3+RFP Miniprep

- Obtained from iGEM Kit of Parts, Kit 5 #23P
- Hydrated with 10 uL PICO water and stored in sequence confirmed box
- According to iGEM, 2-3 ng of DNA provided, so concentration @ .2-.3 ng/uL

Q5 PCR on pSB1C3+RFP 5' end to remove intefering base pairs

- Hydrated 2 primers to 100 uM and made 10 uM stocks in oligos box
- 59 degrees Celsius annealing, 1:45 elongation
- 1 PCR on original DNA @ .2-.3 ng/uL

Inoculated DBAT

- For miniprep Wednesday as we have none left
- 2 x 3 mL LB+KAN tubes
- Inoculated from a 286 plate
- Around 11 PM

Inoculated Rommel 286 Cells

- For competent cell preparation Wednesday as we have little left (Parth continues)
- 2 x 50 mL LB Broth in 250 mL flasks
- Inoculated heavily from labeled plate
- Just past 11 PM

Mini Prep Protocol Zyppy

Introduction

Mini prep using the Zyppy Mini Prep Kit

Materials

- Zyppy Mini Prep Kit
- Cells

Procedure

1. Spin cells down at 3500 rpm for 8 minutes.
2. Add 100 uL of 7X Lysis Buffer (Blue) to 600 UL of E. coli cultrue in a 1.5 ml microcentrifuge tube
3. Mix by inverting the tube 4-6 times and lyse samples for 1-2 min.

00:02:00

4. Add 350 uL of cold Neutralization Buffer (yellow), mix thoroughly.

   Neutralization is complete when sample becomes yellow and precipitate has formed (~1 min)
5. Centrifuge at 11,000 - 16,000 rpm for two minutes.
6. Transfer the supernatant to the spin column
7. Place column into a collection tube and centrifuge at 11,000 rpm for 15 seconds
8. Discard the flow-through and place the column back into the same collection tube
9. Add 200 uL of Endo-Wash Buffer to the column
10. Centrifuge at 11,000 rpm for 15 seconds
11. Add 400 uL of zyppy wash buffer to the column.
12. Centrifuge at 11,000 rpm for 30 seconds
13. Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 uL of Zyppy Elution Buffer directly to the column matrix and let stand for one minute at room temperature.

00:01:00

14. Centrifuge at or less than 11,000 rpm for 15 seconds to elute the DNA

Mini Prep Protocol Zyppy

Introduction

Mini prep using the Zyppy Mini Prep Kit

Materials

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13. Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 uL of Zyppy Elution Buffer directly to the column matrix and let stand for one minute at room temperature.

00:01:00

14. Centrifuge at or less than 11,000 rpm for 15 seconds to elute the DNA

Wk: 9/25 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-09-27 to 2016-09-28

WEDNESDAY, 9/28

Jaydeep (3 - 6:30 PM)

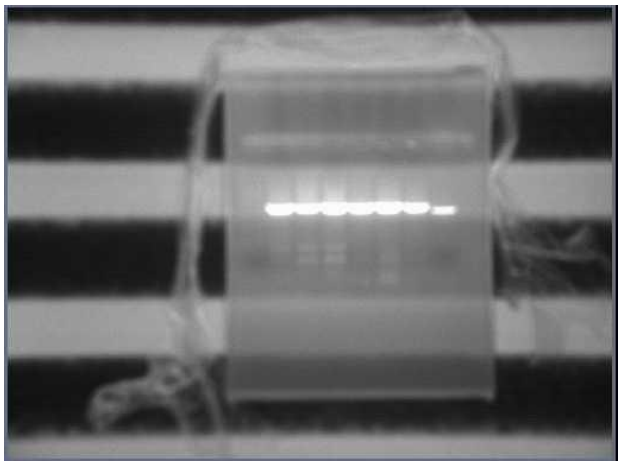
Gel on Tuesday Q5 PCR Products

Table1

	A	B	C	D	E	F	G
1	Well	1	2	3	4	5	
2	Substance	1 kb Ladder	BadA RBS Fix 1 ng	BadA RBS Fix 100 ng	BadA RBS Fix 10 ng	BAPT RBS Fix 1.15 ng	BAPT RBS 23 ng

Key for Gel Q51Round092816

Q51Round092816.jpg



- Bright bands at right lengths across all wells
- Noise least with PCRs with least template DNA (BadA 1 ng, BAPT 1.15 ng) > Proceed to KLD with these PCR products

DBAT Miniprep

Mini Prep Protocol Zyppy

- Cell pellet appeared very small
- 83 ng/uL
- Stored in sequence confirmed box

KLD Reactions - Round 1

- On BadA 1 ng, BAPT 1.15 ng, and pSB1C3+RFP 5' Modification PCR products
- Mixed 2 uL of Kinase, Ligase, and DpnI for 6 uL KLD mix
- Set up reactions in PCR tubes with 1 uL KLD mix, 1 uL ligase reaction buffer, 2 uL PCR product, 6 uL PICO water > mixed
- Let sit at room temperature starting at 5:15 PM

Q5 PCRs on DBAT for RBS Replacement

- Hydrated 2 primers to 100 uM and made 10 uM stocks in oligos box
- 63 degrees Celsius annealing, 2:00 elongation
- 3 PCRs: on original DNA @ 83 ng/ul as well as 1:10 and 1:100 dilutions

Q5 PCR on pSB1C3+RFP 5' Modified to remove intefering base pairs from 3' end

- Hydrated 2 primers to 100 uM and made 10 uM stocks in oligos box
- 59 degrees Celsius annealing, 1:45 elongation
- 3 PCRs on DNA from 5' KLD reaction: original (direct from reaction sitting at 6:15 PM [1.25 hours]) as well as 1:10 and 1:100 dilutions

Jaydeep (10 - 11 PM)

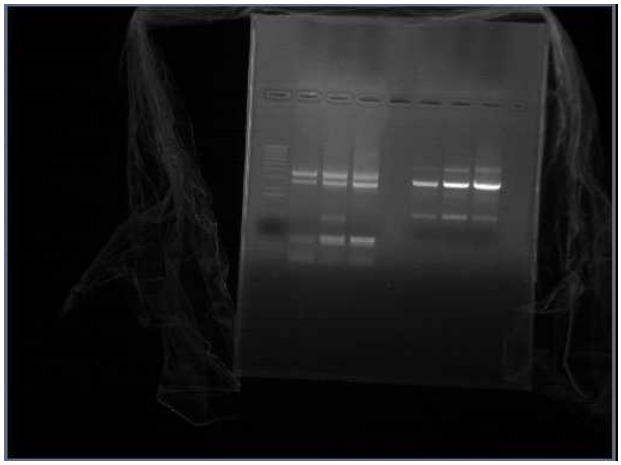
Gel on Evening Q5 PCR Products

Table2

	A	B	C	D	E	F	G
1	Well	1	2	3	4	5	
2	Substance	1 kb Ladder	DBAT RBS Fix 0.83 ng	DBAT RBS Fix 8.3 ng	DBAT RBS Fix 83 ng	Blank	pSB1C3+F 5'+3' Modif 1:100 Dilut 5' KLD

Key for Gel DBATpSB1C3092816

DBATpSB1C3092816.jpg



- Bright bands at right lengths for pSB1C3+RFP fully modified
- DBAT appears to have two PCR products a few hundred base pairs removed > both probably have antibiotic resistance and will grow on plate
- Noise least with PCRs with least template DNA (DBAT 0.83 ng, pSB1C3+RFP 1:100 dilution from 5' KLD) > Proceed to KLD with these PCR products
- **Note that we may have to return to this step and gel extract the correct DBAT band if the following KLD does not work using the PCR product directly**

KLD Reactions - Round 2

- On DBAT 0.83 ng and pSB1C3+RFP Fully Modified PCR products
- Mixed 1 uL of Kinase, Ligase, and DpnI for 3 uL KLD mix
- Set up reactions in PCR tubes with 1 uL KLD mix, 1 uL ligase reaction buffer, 2 uL PCR product, 6 uL PICO water > mixed
- Let sit at room temperature starting at 11:10 PM
- Put in blue box on shelf near fume hood > ready for use at minimum 11:40 PM, great anytime after 2:10 AM

Wk: 10/2 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-10-04 to 2016-10-05

TUESDAY, 10/4

Jaydeep (10:15 PM - 12:45 AM)

Round 3: Electroporation and Transformation of pSmart-HCKan RBS-Fixed DBAT & BadA
1 uL DNA: 35 uL Cells: 250 uL LB Broth recovery media

Table2

	A	B	C
1	BadA: Rommel 286 New Cells	1550 V	654 ms
2	BadA: E. Cloni	1620 V	2.6 ms
3	DBAT: Rommel 286 Old Cells	1580 V	2.2 ms
4	DBAT: E. Cloni	1570 V	2.1 ms

Recovered for 1 hour, 15 min. in new shaker
10 & 100 uL of each transformation plated 12:30 AM

Wk: 10/2 Work Notes

Project: Duke iGEM 2016
Authors: Jaydeep Sambangi
Dates: 2016-10-04 to 2016-10-05

WEDNESDAY, 10/5

Wk: 10/9 Work Notes

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-10-11 to 2016-10-16

TUESDAY, 10/11

Jaydeep & Nisa (6:30 - 9 PM)

Electroporation and Transformation of S.C. pSmart-HCKan RBS-Fixed DBAT
For entry 1: 1 uL DNA: 35 uL Cells: 250 uL LB Broth recovery media
For entry 2: 2 uL DNA: 25 uL Cells: 250 uL LB Broth recovery media: 250 uL PICO (mistake)

Table2

	A	B	C
1	DBAT RBS Fix S.C.: Parth 286	1590 V	2.4 ms
2	DBAT RBS Fix S.C.: Nisa 286	1680 V	3.2 ms

Recovered for 1 hour, 10 min. in new shaker
For entry 1: 10, 100, 140 uL of plated 9 PM
For entry 2: Spun down, 390 uL supernatant discarded, 10 & 100 uL plated 9 PM

Wk: 10/9 Work Notes

Project: Duke iGEM 2016
Authors: Ben Hoover
Dates: 2016-10-11 to 2016-10-16

SUNDAY, 10/16

Ben replated DBATx1C3 and BadAx1C3 transformations onto cm plates @ 1:15pm